

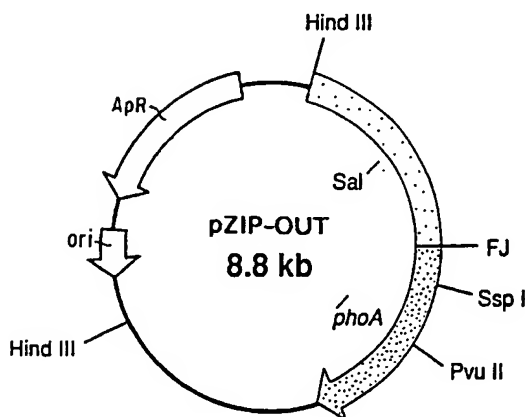


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(54) Title: MEMBRANE EXPRESSION OF HETEROLOGOUS GENES**(57) Abstract**

The invention relates to nucleic acid segments useful in the construction of expression vectors for expression of heterologous polypeptides directed to particular areas of the host cell. Selected constructs direct production of polypeptides to the outer membrane surface of the cell. Other constructs direct expression of heterologous polypeptides to the inner membrane/periplasm of the host cell. Transformed host cells are potentially useful for the production of vaccines or immunogens elicited in response to antigens expressed on the outer membranes of the host cells.



-333
5'- AAATCCTG/GAAACCGATTGCGCCCTTATAACTATTGTTCAGATA

ACGTTCTGACGGTGTGTAAAAACATGGCGCTCATTTCTCTGTAGTTGGAGTTAAT

met lys lys phe tyr ser cys leu pro val phe leu ile gly

ATG AAA AAA TTT TAT AGC TGT CTT CCT GTC TTT TTA CTG ATC GGC

cys pro ser thr val ala ser thr gln

TGT GCT CCT GAC TCT TAT ACA CAA GTA GCG TCC TGG ACG GAA

-1550L

pro phe pro phe cys pro val leu gln asn arg

CCT TTC CCG TTT TGC CCT GTT CTG GAA AAC CCG // -3'

phoA

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DESCRIPTIONMEMBRANE EXPRESSION OF HETEROLOGOUS GENES

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BACKGROUND OF THE INVENTIONField of the Invention

The invention relates generally to the exportation
10 of heterologous polypeptides to discrete regions of a
host cell in which it is expressed, to nucleic acid
sequences encoding exportation polypeptides, to the
preparation of membrane embedded epitopes of immunogenic
antigens, and to vectors constructed with selected
15 exportation sequences. More particularly, localized
expression of polypeptides may be obtained by providing
exportation signals encoded by segments of the disclosed
nucleic acids that provide for exportation of expressed
heterologous polypeptides to the inner
20 membrane/periplasmic space or the outer membrane surface
of a host cell.

Description of Related Art

25 Recombinant gene technology has been extensively
investigated in the context of expression of foreign
proteins in host cells which harbor recombinant genes,
typically bacterial host cells. Such expression is
desirable for producing high value proteins, immunogenic
30 polypeptides, and in obtaining hybrid proteins that are
otherwise difficult to synthesize.

Of particular interest is vaccine development. It
is potentially feasible to prepare protective vaccines
35 from epitopes of known antigens of eukaryotic, viral or
prokaryotic pathogens by taking advantage of the
synthetic capacities of transformed host cells. Examples

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include tumor specific proteins which might be expressed and utilized to stimulate an immune response. Oral vaccines have stimulated research because of the ease of administration and, more importantly, in some instances the unsatisfactory protection afforded from parenteral injection. Vaccination against cholera, for example, gives short-term protection, thus provoking developmental work toward an oral vaccine that would presumably stimulate mucosal intestinal immunity more efficiently (Sanchez et al., 1990).

Salmonella strains are being studied experimentally as particularly attractive candidates for producing oral live vaccines. Attenuated strains have been shown to elicit immune responses in several animal species (Strugnell et al., 1990) and apparently can be highly immunogenic in the host. Humoral antibody responses including local secretory antibody and cellular immune responses have been observed after oral intake (Dougan et al., 1986). Attenuated mutants have been identified via screening procedures such as *TnphoA* mutagenesis, which exclude elimination of mutations in nonsecreted proteins (Miller et al., 1989). However, *TnphoA* methods only indicate assessment of integration of the transposon into a gene for a secreted or cytoplasmic protein.

Protein expression systems have been developed from *Salmonella* strains. A cloning vector useful for integrating DNA into the *aroC* gene on *Salmonella* chromosomes was used to direct expression of heterologous antigens such as tetanus toxin fragment C and *Treponema pallidum* lipoprotein (Strugnell et al., 1990). In some cases, heterologous polypeptide gene products orally administered have elicited a serum antibody response, as for example, the cholera toxin B subunit protein expressed from a recombinant *Yersinia enterocolitica* strain (Sory and Cornelis, 1990). Unfortunately, while

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antibodies were detected in sera of challenged mice, the response was variable and was directed toward polymeric forms of cholera toxin B.

5 It is recognized that cytoplasmic proteins may not produce a high immunogenic response and heterologous proteins from recombinant DNA molecules expressed cytoplasmically often exhibit a diminished antibody reactivity (Sanchez, et al., 1990). Thus surface
10 expressed epitopes of bacteria are expected generally to elicit the greatest humoral response; however, factors controlling surface expression of heterologous proteins have not been defined and there is no way to assure that any given fusion protein will localize to a host cell
15 membrane surface.

Vaccines are the most cost effective medical intervention known to prevent disease. However, effective vaccines are available for relatively few
20 diseases. Successful immunization against infectious organisms often requires a multicomponent host immune response against a variety of antigenic determinants. Orally administered vaccines, especially live attenuated vaccines, induce specific cell-mediated effector
25 responses and elicit secretory IgA (sIgA) responses. SIgA is important because of its effectiveness at mucosal surfaces. SIgA production and cell effector responses are mediated through the delivery of antigens to gut-associated lymphoid tissue (GALT). Stimulation of GALT
30 can lead to effective cell and humoral defense at all mucosal surfaces and provide systemic protection (1,2).

To deliver antigens to GALT, investigators have developed avirulent and virulence-attenuated *Salmonella*
35 strains. Aromatic dependent (aroA (3)), phoP (4), gale (5), and cya/crp (6) *Salmonella* mutants have been reported to interact with GALT in the lamina propria and

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stimulate an immune response. While it is clearly desirable to use avirulent *Salmonella* strains as carriers for plasmids which express protective antigens of other pathogens on their surface, it is clear that improvements
5 are needed to develop protective vaccines based on this system.

The use of attenuated *Salmonella* strains to express heterologous antigens and stimulate GALT is being
10 extensively investigated. In some studies, detectable levels of specific mucosal and serum antibodies to the heterologously expressed antigen have been observed (7-10). However, in general results with most antigens have been variable.

15 It is generally believed that the export of heterologous epitopes to the *Salmonella* cell surface enhances their immunogenicity (11). Investigators have used recombinant DNA methods to express heterologous
20 epitopes as inserts in *Salmonella* flagellin (9) and the *lamB* encoded polypeptide of *E. coli* (10). In these studies, a significant antibody response to the heterologous surface-expressed epitopes was observed. A
25 limitation of these systems is the relatively small number of epitopes which can be inserted into the *lamB* and flagellin genes. This is important as single (or few) epitopes may not result in the broad-based immune response which characterizes today's most successful
30 vaccines.

There is clearly a need to develop effective systems to elicit antibody response and in particular to provide methods of exporting heterologous polypeptides to the surface of appropriate host cells. Antigenic peptides
35 expressed on bacterial host cell surfaces may be significant in developing vaccines to such important antigens as cholera B subunit toxin and HIV antigens.

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SUMMARY OF THE INVENTION

The present invention addresses one or more of the foregoing or other problems associated with methods of controlling surface expression of heterologous polypeptides in a host cell and provides in particular a method of directing exported polypeptides to outer cell membrane surfaces or to inner membrane/cytoplasmic regions. The invention includes nucleic acid segments useful for preparing expression vectors. Such vectors are suitable for expressing and directing heterologous polypeptides exported to selected areas of the host cell. Transformed cells with surface expressed antigens or epitopic regions are expected to be useful as immunogens producing an effective immune response.

The nucleic acid segments of the present invention encode amino acid sequences associated with particular targeting of fused heterologous polypeptides to particular areas of a transformed host cell. It has been found for example that nucleic acid segments defined by SEQ ID NO:1 encode a polypeptide product which when fused to a heterologous polypeptide will direct that polypeptide to the outer membrane of a bacterial cell. By heterologous polypeptide is meant any polypeptide other than those normally associated SEQ ID NO:1. It is of course understood that such localizing capabilities are realized under conditions when the exportation polypeptide is incorporated into a suitable expression vector and an appropriate cell host is transformed with that vector. A preferred embodiment of the DNA segment is defined by SEQ ID NO:1. This sequence fused to a *phoA* sequence encodes a 46 Kda polypeptide.

The present invention also includes nucleic acid segments encoding amino acid sequences associated with the transport of heterologous polypeptides to the

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bacterial inner membrane periplasmic space. Particular
embodiments of these sequences are included in the
nucleic acid sequences defined in SEQ ID NO:2. A
preferred inner membrane periplasmic space directing
5 polypeptide is a 55 Kda polypeptide encoded by the gene
sequence illustrated in Figure 3 and defined in SEQ ID
NO:2. This preferred embodiment includes gene sequences
encoding part of the alkaline phosphatase gene, however,
other heterologous genes could be used in place of
10 alkaline phosphatase.

While particular nucleic acid sequences have been
defined it is nevertheless contemplated that nucleic acid
sequences will be found to vary. It is expected that
15 analogous sequences with similar functions may be found
in other gram-negative bacteria such as *E. coli*.

In certain particular embodiments, the invention
concerns expression vectors that are constructed to
20 include any of the DNA segments herein disclosed. Such
DNA may be fused directly with a gene of interest and
used in an expression system to produce heterologous
polypeptides as hybridization probes for, e.g.,
identifying related sequences, as primers or even as
25 building blocks for the construction of mutant or variant
sequences. A particularly useful application of the DNA
segments of this invention is to achieve directed
expression of heterologous polypeptides. Depending on
the DNA segment selected, polypeptides will be expressed
30 on the inner membrane periplasmic space, the outer
membrane of the host cell, or on the surface of the outer
membrane of the host cell.

In particular embodiments, the pZIP plasmids of
35 Figure 2 and Figure 3 have been constructed. Depending
on the plasmid selected, fusion polypeptides are exported
to the inner membrane/periplasmic space or to the outer

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membrane of the host cell. In a preferred embodiment, pZIP-OUT directs the export of fusion polypeptides to the outer membrane and may also direct a heterologous peptide to the external surface of a gram-negative host cell.

5 pZIP-OUT is a vector which expresses bipartite fusion which includes a DNA segment capable of exporting the fusion product to the external membrane of a gram-negative cell. The other part of the chimeric gene is a *phoA* gene segment lacking signal and expression segments.

10 A variety DNA segments may be inserted into the *phoA* segment at suitable restriction sites to create a tripartite fusion.

Yet another preferred embodiment is the pZIP-IN

15 plasmid shown in Figure 3. This plasmid directs the export of polypeptides to the inner membrane/periplasmic space. The construction of the plasmid is bipartite. Part of the alkaline phosphatase gene lacking signal and expression sequences is fused with a DNA sequence that

20 contains an exportation sequence capable of directing its fusion polypeptide to an inner membrane/periplasmic space. There are several restriction sites in the *phoA* gene segment into which foreign DNA or fragments of DNA may be inserted.

25

Other components of either of these plasmids may include, in addition to the export specifying sequences, resistance genes such as ampicillin or tetracycline resistance genes. In addition an *E. coli phoA* gene may

30 be fused in frame with expression directing DNA sequences, such as that used to construct the pZIP-IN and pZIP-OUT plasmids. pZIP-IN additionally encodes a kanamycin resistance gene. An advantage of using the *phoA* fusion is that there are various restriction sites

35 within the *phoA* gene facilitating the fusion of heterologous gene sequences in frame with *phoA* and the export specifying sequences.

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Expression vectors may also include a gene encoding a detectable polypeptide. Typical examples of reporter genes encoding detectable polypeptides include β -lactamase and alkaline phosphatase genes. Reporter genes
5 may be conveniently fused in frame downstream of the disclosed nucleic acid sequences with or without other DNA fragments/segments. Moreover, restriction sites in the gene sequence of the reporter gene may be used for insertion of a desired DNA fragment(s).

10

Recombinant vectors such as those described are particularly preferred for transforming bacterial host cells. Several types of bacterial host cells may be employed, most preferred being gram-negative cells such
15 as *E. coli*, *Salmonella* and the like.

Transformed cells may be selected using various techniques including screening by differential hybridization, identification of fused reporter gene
20 products, resistance markers, anti-antigen antibodies, and the like. After identification of an appropriate clone it may be selected and cultivated under conditions appropriate to the circumstances, as for example, conditions favoring expression.

25

Another aspect of the invention is a method of preparing heterologous polypeptides. The method generally involves preparing one or more of the recombinant vectors herein disclosed, transforming a host
30 cell with the recombinant vector, then selecting a vector containing host cell clone and finally isolating from the clone the desired polypeptide which will be a heterologous protein. Examples of useful proteins that might be used in preparing the recombinant vector include
35 alkaline phosphatase, cholera toxin B subunit, fragments of these proteins, or any other desired proteins.

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Depending on the particular recombinant vector selected for transforming a host cell, recombinant heterologous polypeptides will be expressed in different compartments of the cell. For those heterologous polypeptides expressed in the inner membrane or periplasmic space isolation of the heterologous polypeptide may be affected by cell lysis and other procedures utilized in the isolation of a desired fusion protein. Heterologous fusion proteins exported to the outer membrane of the host cell may be isolated from the outer membrane directly. Typical procedures include separation of inner and outer cell membranes and then isolation of the fusion polypeptide from membranous material.

15

In a preferred embodiment, antigenic proteins are expressed on the surface of the host cell. Selected epitopes of eukaryotic viral or prokaryotic pathogens expressed on the surface of a host cell may be used for vaccine development. Tumor specific genes could be expressed and utilized to stimulate an immune response. Whole cells expressing immunogenic epitopes might be used for agglutination-based screening tests. Surface expressed polypeptides of other organisms might be identified by screening recombinant libraries for specific surface expressed polypeptides. In another preferred embodiment, cholera toxin B subunit may be expressed on the surface of a *Salmonella* harboring the pZIP-OUT plasmid vector hereinabove described. When expressed from *Salmonella* strain TA2362 harboring plasmid pRSP18, cholera toxin B subunits agglutinated in the presence of specific antibody, indicating exposure of epitopic regions on the external membrane surface of formalin-fixed cells.

35

Another aspect of the invention involves the preparation of vaccines. Antigens or epitope(s) are

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selected and a gene encoding these moieties is inserted into one or more of the recombinant vectors disclosed. Appropriate host cells are transformed and after screening for transformants one is selected which
5 expresses the antigen or epitopes for which a vaccine is desired. Vaccines may then be prepared by a variety of methods. Antigens on the surface of appropriate host cells may be safely administered orally. For example, attenuated *Salmonella* orally administered could stimulate
10 an immune response on gut mucosa. Alternatively, whole cells or cell fragments containing the membrane-bound antigen may be suitably injected into a mammal to generate an immune response. In any event, it is expected that the immunogenicity of an antigen or epitope
15 may be significantly enhanced when expressed on the surface of a bacterial cell.

In both immunodiagnostics and vaccine preparation, it is often possible and indeed more practical to prepare
20 antigens from segments of a known immunogenic protein or polypeptide. Certain epitopic regions may be used to produce responses similar to those produced by the entire antigenic polypeptide. Often however responses to epitopic regions are not so strong as responses to the
25 entire polypeptide. However, surface expression of these epitopes may generate an enhanced immune response.

In other embodiments, the invention concerns primers capable of priming amplification of selected portions of
30 disclosed DNA segments. Primers hybridize to DNA and serve as initiation sites for synthesis of a portion of the gene. Nucleotide primers are designed to bind at separate sites on opposing duplex strains thereby defining the intervening sequence as the portion to be
35 amplified. Nucleic acid molecules to be employed as primers whether DNA or RNA will generally include at least a 10 nucleotide segment of the nucleic acid

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sequence of SEQ ID NO:1 or SEQ ID NO:2. The 10 base pair size is selected as a general lower limit in that sizes smaller than 10 bases hybridization stabilization may become a problem. However, as the size of the primer decreases too much below 7-8 bases, non-specific hybridization may occur with other genes having complimentary sequences over short stretches.

Primers may be utilized for several purposes. For example, primers may be used to amplify selected portions of the disclosed DNA segments. Certain primer combinations may more efficiently generate DNA encoding polypeptides that more effectively target to inner or outer membranes. Additionally, primers prepared from the disclosed DNA may be used to amplify regions of DNA from other related organisms in order to identify similar targeting sequences. Once amplified products are obtained probes which referred to nucleic acid molecules employed to detect DNA sequences through hybridization procedures may be employed to detect and isolate selected DNA fragments. Like primers, probes may be DNA or RNA and are generally of similar size usually including at least a 10 nucleotide segment or more, often of 220 or 21 base pairs. Probes may be labeled, for example, by radio labeling, to assist in identification of nucleic acid sequences.

As part of the invention, kits useful for the expression of fusion proteins are also envisioned comprising separate containers, each having suitably aliquoted reagents for performing the foregoing methods. For example, the containers may include one or more vectors, examples being the vectors of claim 19, particular embodiments of which are shown schematically in Figures 4 and 5. Suitable containers might be vials made of plastic or glass, various tubes such as test tubes, metal cylinders, ceramic cups or the like.

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Containers may be prepared with a wide range of suitable aliquots, depending on applications and on the scale of the preparation. Generally this will be an amount that is conveniently handled so as to minimize handling and subsequent volumetric manipulations. Most practitioners will prefer to select suitable endonucleases from common supplies usually on hand; however, such restriction endonucleases could also be optionally included in a kit preparation.

10

Vectors supplied in kit form are preferably supplied in lyophilized form, although such DNA fragments may also be taken up in a suitable solvent such as ethanol, glycols or the like and supplied as suspensions. For most applications, it would be desirable to remove the solvent which for ethanol, for example, is a relatively simple matter of evaporation.

15

BRIEF DESCRIPTION OF THE DRAWINGS

20

Figure 1 illustrates the cloning of *phoA* gene fusion from *TnphoA* insertion mutants and construction of tribrid gene fusions. *TnphoA* is a derivative of *Tn5* which encodes *E. coli* alkaline phosphatase, minus the signal sequence and expression signals, inserted into the left IS50L element (21). Random transposition of *TnphoA* results in an active insertion only when the *phoA* gene sequence is fused in frame downstream of the promoter and export signals of a target gene (A). The point at which the *phoA* sequence joins the target gene is referred to as the fusion joint (FJ). The remaining portion of the gene begins at the distal joint (DJ). Utilizing restriction enzymes which cut either downstream of the kanamycin resistance gene (e.g., *Bam*HI) or the *phoA* gene sequence (e.g., *Hind*III), allows cloning of *phoA* gene fusions (if the target gene is not also restricted ("R")). Plasmids carrying *phoA* gene fusions can then be used as exposition

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vectors (B). The *SspI* and *PvuII* restriction sites in *phoA* provide blunt ended sites at which in frame insertions (IF) of a gene of interest (GOI) can be inserted. The GOI must also be consistent with the *phoA* frame at the insertion site. The resulting tribrid gene fusions contain the expression and export signals of the target gene fused in frame with the *phoA* and GOI sequences.

Figure 2B shows the DNA sequence across the *Salmonella::phoA* fusion joint in pZIP-OUT. Dideoxy sequencing (Sequenase 2.0 USB Biochemicals) was used to determine the 353 base pairs (bp) upstream of the *Salmonella::phoA* fusion joint. A single open reading frame (ORF) which was in frame with that of the IS50L/*phoA* sequence was observed. A stop codon in this ORF was observed at position -99. Multiple stop codons in all reading frames were present in sequences -150 to -200. Two putative translation start codons (AUG) were present at positions -84 and -51. A putative Pribnow box (=) was present at position -120. The predicted amino acid sequence of the coding region is shown above the nucleotide sequence. The IS50L and the beginning of the *phoA* derived sequences are underlined.

Figure 2 schematically shows plasmid pZIP-OUT contains a 4.5 Kb *HindIII* chromosomal fragment from invasion-attenuated *S. typhimurium* Tn*phoA* insertion mutant TAP 43 inserted into pBR322 at the *HindIII* site. It expresses a 46 Kd PHOA fusion protein which localizes to the outer membrane.

Figure 3 shows plasmid pZIP-IN which contains a *BamHI* chromosomal fragment from *S. typhimurium* Tn*phoA* insertion mutant TAG 28, inserted into pBR322 at the *BamHI* site. It expresses a 55 kd PhoA fusion protein which localizes to the inner membrane.

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Figure 4 is an immunoblot analysis of *Salmonella* membrane preparations using mouse anti-alkaline phosphatase. *S. typhimurium* TA 2362 harboring pBR322 showed no reaction in the total envelope (TE). TA 2362
5 harboring pZIP-OUT showed a 46 Kd PHOA fusion in the TE and after separation of the inner and outer membrane by treatment with 0.5% sarkosyl, the majority of the fusion protein was associated with the outer membrane (OM). TA
10 2362 harboring pZIP-IN showed a 55 Kd PhoA fusion protein in the TE and after separation of the inner and outer membrane by treatment with 0.5% sarkosyl, the majority of the fusion protein was found associated with the inner membrane (IM). All lanes were loaded with membrane
15 preparations from an equal amount of cells.

Figure 5 is an immunoblot analysis of urea extracts (SURF) using anti-alkaline phosphatase as the primary antibody. *S. typhimurium* TA 2362 harboring pBR322 showed
20 no reacting polypeptides to the alkaline phosphatase antibodies. TA 2362 harboring pZIP-OUT showed a PhoA fusion at 46 Kd. TA 2362 harboring pZIP-IN showed no reacting polypeptides with the same antisera. Lanes were loaded with an equivalent amount of extract prepared from
25 an equivalent number of whole cells.

Figure 6 shows the derivation of plasmid pRSP18 from pZIP-OUT in which the final 294 base pairs of *ctxB* have
been inserted in frame (IF) with the *phoA* gene sequence at the PvuII site. The *ctxB* gene sequence is from
30 pRIT10810 which encodes the entire *ctxB* gene (22).

Figure 7 shows the derivation of plasmid pIMB13 from pZIP-IN in which the final 294 base pairs of *ctxB* have
been inserted in frame (IF) with the *phoA* gene sequence at the *SspI* site. The *ctxB* gene sequence is from
35 pRIT10810 which encodes the entire *ctxB* gene (22).

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Figure 8 is a schematic representation of the CtxB fusion from pRSP18 and pIMB13 that results in exportation of the 32 kDa CtxB protein to the outer and inner membranes, respectively.

5

Figure 9 is an immunoblot analysis of urea extracts (SURF) using affinity purified anti-CTB as the primary antibody. *S. typhimurium* TA 2362 harboring pRIT10810 which encodes cytoplasmically expressed CTB showed no reaction. TA 2362 harboring pRSP18 showed a CTB tribrid fusion protein at 32 Kd. TA 2362 harboring pIMB13 showed no reactivity to anti-CTB antibodies. Lanes were loaded with equal amounts of extract from equivalent numbers of whole cells.

15

Figure 10 is a proposed protocol for insertion of a fragment of HIV gp160 gene into pZIP-OUT.

Figure 11 shows the sequence of export specific signal in pZIP-IN. Promoter and regulatory sequences are underlined. IS50L and *phoA* sequences from pZIP-IN are shown. The ORF is shown in capital letters.

20

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

25

The present invention relates to nucleic acid segments encoding particular polypeptides capable of forming fusion proteins that export to particular areas of a host cell. These nucleic acid segments are useful in constructing vectors that allow expression of heterologous proteins in appropriately transformed host cells. Polypeptides may be localized within the inner membrane/periplasmic space or on the outer membrane surface. Antigens or epitopic regions of antigens localized on host cell membranes have particular potential for vaccine development and antibody production.

35

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A heterologous gene expression system has been developed which utilizes a virulence-attenuated *Salmonella* as a carrier for a plasmid expression system (pZIP-OUT) which can direct the products of large segments of heterologous genes to the outer membrane (Fig. 2). Recombinant DNA techniques are utilized to fuse the reading frame of the gene to be expressed with *Salmonella* export specifying sequences, Figure 1. Several cloning sites are possible which allow maintenance of the proper reading frame and produce tribrid fusion polypeptides which contain *Salmonella* export specifying sequences, the heterologous gene sequences and *phoA* gene sequences. Recombinants which export the tribrid fusion protein are selected through the loss of *phoA* activity and appearance of the predicted fusion polypeptide on the surface of the outer membrane. A tribrid fusion has been constructed which encodes virtually the entire cholera toxin B subunit (*ctxB*) gene, Figure 6, and evaluated its subcellular localization in *Salmonella*. This fusion polypeptide is expressed on the *Salmonella* surface as evidenced by: 1) agglutination of tribrid fusion expressing strains by anti-CTB antiserum, 2) localization of the fusion polypeptide in the outer membrane, and 3) the presence of the fusion polypeptide in cell surface preparations.

The DNA of the present invention was isolated from *Salmonella typhimurium*, strain TAP43, an invasion attenuated strain. Invasion attenuated refers to species which have lost one or more virulence factors affecting the efficiency by which *Salmonella* invades epithelial cells. Isolation of an attenuated strain of *Salmonella* was considered useful in developing the present invention because such strains may be used to deliver heterologous antigens to the gut of an animal. *Salmonella* given orally tends to establish an infection in the intestinal mucosa, leading to an immune response. The presence of a

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desired antigen is expected to stimulate a response to that species, as well as to the *Salmonella* or other host antigens.

5 The approach to screening for protein export signals was to use alkaline phosphatase fusions based on the TnphoA transposon system reviewed by Manoil et al. (1990). TnphoA is a transposon derivative of Tn5 in the phoA gene which lacks a promoter, translation initiation
10 site, signal sequence DNA and the first five amino acids of its protein. When the transposon, TnphoA, inserts into a foreign gene in the correct orientation and reading frame, gene fusions are generated, coding for hybrid proteins which have alkaline phosphatase activity
15 if transported beyond the inner membrane. Detection of such activity is generally accomplished with an alkaline phosphatase indicator dye, allowing visualization of colored colonies for successful gene fusions that lead to export of heterologous gene products.

20
Part of the present invention contemplates vaccine preparation and use. In general, it is contemplated that antigens, or epitopes of antigens, will be readily expressed in localized regions of a host cell using the
25 methods disclosed. Expression vectors incorporating the DNA segment encoding exportation polypeptides directing products to a host cell outer membrane surface are expected to be particularly useful. Epitopic regions of antigens, well exposed at a membrane surface, may elicit
30 high immunogenic responses, providing a route to vaccines or antibody production.

General concepts related to methods of vaccine preparation and use are discussed as applicable to
35 preparations and formulations with antigens, epitopes or subfragments of such antigens obtained from various

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sources; for example, cholera B toxin subunit and the like.

Vaccine Preparation and Use

5

Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; 10 and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as injectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may 15 also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and 20 combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or adjuvants which enhance the effectiveness of the vaccines.

25

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of 30 administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active 35 ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of

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mannitol, lactose, starch, magnesium stearate, sodium
saccharine, cellulose, magnesium carbonate, and the like.
These compositions take the form of solutions,
suspensions, tablets, pills, capsules, sustained release
5 formulations or powders and contain 10-95% of active
ingredient, preferably 25-70%.

The proteins may be formulated into the vaccine as
neutral or salt forms. Pharmaceutically acceptable salts
10 include acid addition salts (formed with the free amino
groups of the peptide) and which are formed with
inorganic acids such as, for example, hydrochloric or
phosphoric acids, or such organic acids as acetic oxalic,
tartaric, mandelic, and the like. Salts formed with the
15 free carboxyl groups may also be derived from inorganic
bases such as, for example, sodium, potassium, ammonium,
calcium, or ferric hydroxides, and such organic bases as
isopropylamine, trimethylamine, 2-ethylamino ethanol,
histidine, procaine, and the like.

20

The vaccines are administered in a manner compatible
with the dosage formulation, and in such amount as will
be therapeutically effective and immunogenic. The
quantity to be administered depends on the subject to be
25 treated, including, e.g., the capacity of the
individual's immune system to synthesize antibodies, and
the degree of protection desired. Precise amounts of
active ingredient required to be administered depend on
the judgment of the practitioner. However, suitable
30 dosage ranges are of the order of several hundred
micrograms active ingredient per vaccination. Suitable
regimes for initial administration and booster shots are
also variable but are typified by an initial
administration followed by subsequent inoculations or
35 other administrations.

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The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These are believed to include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the size of the host.

Various methods of achieving adjuvant effect for the vaccine include use of agents such as aluminum hydroxide or phosphate (alum), commonly used as 0.05 to 0.1 percent solution in phosphate buffered saline, admixture with synthetic polymers of sugars (Carbopol) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively. Aggregation by reactivating with pepsin treated (Fab) antibodies to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil vehicles such as mannide mono-oleate (Aracel A) or emulsion with a 20 percent solution of a perfluorocarbon (Fluosol-DA) used as a block substitute may also be employed.

In many instances, it will be desirable to have multiple administrations of the vaccine, usually not exceeding six vaccinations, more usually not exceeding four vaccinations and preferably one or more, usually at least about three vaccinations. The vaccinations will normally be administered from two to twelve week intervals, more usually from three to five week intervals. Periodic boosters at intervals of 1-5 years, usually three years, will be desirable to maintain protective levels of the antibodies. The course of the immunization may be followed by assays for antibodies for

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the supernatant antigens. The assays may be performed by labeling with conventional labels, such as radionuclides, enzymes, fluorescers, and the like. These techniques are well known and may be found in a wide variety of patents, such as U.S. Patent Nos. 3,791,932; 4,174,384 and 3,949,064, as illustrative of these types of assays.

The invention also contemplates the use of disclosed nucleic acid segments in the construction of expression vectors or plasmids and use in host cells. The following is a general discussion relating to such use and the particular considerations in practicing this aspect of the invention.

15 Host Cell Cultures and Vectors

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and constructing the vectors useful in the invention. For example, in addition to the particular strains mentioned in the more specific disclosure below, one may mention by way of example, strains such as *E. coli* K12 strain 294 (ATCC No. 31446), *E. coli* B, and *E. coli* X 1776 (ATCC No. 31537). These examples are, of course, intended to be illustrative rather than limiting.

Other prokaryotes may also be preferred for expression. The aforementioned strains, as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325), bacilli such as *Bacillus subtilis*, or other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *Pseudomonas* species may be used.

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host cell are used in connection with

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these hosts. The vector ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a
5 plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must
10 also contain, or be modified to contain, promoters which can be used by the microorganism for expression.

Those promoters most commonly used in recombinant DNA construction include the β -lactamase (penicillinase)
15 and lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and
20 utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally into plasmid vectors (Siebwenlist et al., 1980). Certain genes from prokaryotes may be expressed efficiently in *E. coli* from
25 their own promoter sequences, precluding the need for addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces*
30 *cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb et al., 1979; Kingsman et al., 1979;
35 Tschemper et al., 1980). This plasmid already contains the *trp1* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in

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tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzman et al., 1980) or other glycolytic enzymes (Hess et al., 1968; Holland et al., 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue

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culture) has become a routine procedure in recent years (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers *et al.*, 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *HindIII* site toward the *BglI* site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

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Also contemplated within the scope of the present invention is the use of the disclosed DNA as a hybridization probe. While particular examples are provided to illustrate such use, the following provides
5 general background for hybridization applications taking advantage of the disclosed nucleic acid sequences of the invention.

Nucleic Acid Hybridization Embodiments

10

In certain aspects, the DNA sequence information provided by the invention allows for the preparation of relatively short DNA (or RNA) sequences having the ability to specifically hybridize to *S. typhimurium* gene
15 sequences. In these aspects, nucleic acid probes of an appropriate length are prepared based on a consideration of the sequence, e.g., as shown SEQ ID NO:1 and SEQ ID NO:2 or derived from flanking regions of these genes. The ability of such nucleic acid probes to specifically
20 hybridize to the *S. typhimurium* gene sequences lend them particular utility in a variety of embodiments. The probes can be used in a variety of diagnostic assays for detecting the presence of pathogenic organisms in a given sample. However, other uses are envisioned, including
25 the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructs.

To provide certain of the advantages in accordance
30 with the invention, the preferred nucleic acid sequence employed for hybridizations or assays includes sequences that are complementary to at least a 10 to 40, or so, nucleotide stretch of the selected sequence, such as that shown in Figure 1 or Figure 2, SEQ ID NO:1 or SEQ ID
35 NO:2. A size of at least 10 nucleotides in length helps to ensure that the fragment will be of sufficient length to form a duplex molecule that is both stable and

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selective. Molecules having complementary sequences over stretches greater than 10 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. Thus, one will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 20 nucleotides, or even longer where desired. Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102, or by introducing selected sequences into recombinant vectors for recombinant production.

15

Accordingly, the nucleotide sequences of the invention are important for their ability to selectively form duplex molecules with complementary stretches of *S. typhimurium* gene segments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of the probe toward the target sequence. For applications requiring a high degree of selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, for example, one will select relatively low salt and/or high temperature conditions, such as provided by 0.02 M-0.15 M NaCl at temperatures of 50°C to 70°C. These conditions are particularly selective, and tolerate little, if any, mismatch between the probe and the template or target strand.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template, less stringent hybridization conditions are called for in order to allow formation of the heteroduplex. In these

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circumstances, one would desire to employ conditions such as 0.15 M-0.9 M salt, at temperatures ranging from 20°C to 55°C. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

10

The present invention is envisioned as useful in the cloning of nucleic acids encoding certain exportation polypeptides. Identification of other exportation polypeptides in addition to the 46 kDa and 55 kDa proteins should be possible using methods analogous to those disclosed herein. One method would be to produce a cDNA library using mRNA obtained from mutant *S. typhimurium* strains. Although the production of cDNA libraries from bacteria is not commonly done because of the usual absence of poly-A tails on prokaryotic messages, a cDNA library could be constructed from *S. typhimurium* mRNA.

A method of preparing variants of the *S. typhimurium* exportation polypeptides is site-directed mutagenesis. This technique is useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, derived from the 46 kDa or 55 kDa protein sequence, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient

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number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about
5 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific
10 mutagenesis is well known in the art as exemplified by publications (Adelman et al., 1983). As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed
15 mutagenesis include vectors such as the M13 phage (Messing et al., 1981). These phage are readily commercially available and their use is generally well known to those skilled in the art.

20 In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector which includes within its sequence a DNA sequence which encodes an export polypeptide. An oligonucleotide primer bearing the desired mutated
25 sequence is prepared, generally synthetically, for example by the method of Crea et al. (1978). This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the
30 synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli*
35 cells, and clones are selected which include recombinant vectors bearing the mutated sequence.

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The preparation of sequence variants of the selected exportation polypeptide gene using site-directed mutagenesis is provided as a means of producing potentially useful exportation species and is not meant to be limiting as there are other ways in which sequence variants of the exportation polypeptide gene may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents to obtain sequence variants (see, e.g., a method described by Eichenlaub, (1979) for the mutagenesis of plasmid DNA using hydroxylamine).

Isolation of *Salmonella* DNA segments was accomplished by isolation of DNA fragments containing the *phoA* gene *phoA* fusions. *TnphoA* is a derivative of *Tn5* which encodes *E. coli* alkaline phosphatase, minus the signal sequence and expression signals, inserted into the left IS50L element. Random transposition of *TnphoA* results in an active insertion only when the *phoA* gene sequence is fused inframe downstream of the promoter and export signals of a target gene A, Figure 1. Plasmids containing *phoA* gene fusions can then be used as exposition vectors, Figure 1, (B). The *SSP1* and the *PvuII* restriction sites in *phoA* are blunt ended sites at which inframe insertions (IF) of a gene of interest (GOI) can be inserted. The resulting tribrid gene fusions, shown as C in Figure 1, contain the expression and export signals of the target gene fused inframe with the *phoA* and GOI sequences.

30

Figure 1 is a schematic representation of typical *phoA* fusions and illustrating cloning of successful fusions. The point at which the *phoA* sequence joins the target gene is referred to as the fusion joint (FJ). The remaining portion of the gene begins at the distal joint (DJ). Utilizing restriction enzymes which cut either downstream of the kanamycin resistance gene (e.g., *BamHI*)

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-30-

or the *phoA* gene sequence (e.g., *Hind*III) allows cloning of *phoA* gene fusions, provided the target gene is not cleaved ("R"). The fusion joint, including all the *phoA* gene fusions and upstream *Salmonella* sequences, were
5 cloned into the *Hind*III or *Bam*HI site of pBR322, Figures 2 and 3. Plasmids containing *phoA* gene fusions were then used as expression vectors. Cells produced fusion polypeptides that had alkaline phosphatase activity, indicated by the formation of blue colonies on agar
10 supplemented with the indicator dye (5-bromo-4-chloro-3-indolylphosphate).

The following examples are intended to illustrate the practice of the present invention and are not
15 intended to be limiting. Although the invention is demonstrated with nucleic acid segments isolated from a strain of *Salmonella*, similar functions may be obtained from nucleic acid segments from other *Salmonella* strains and even other microorganisms. The nucleic acid
20 sequences identified and the corresponding encoded polypeptides are useful in developing methods of producing a wide variety of heterologous proteins as well as expression vectors for localizing polypeptides in selected areas of a host cell.

25

EXAMPLE 1

The following illustrates construction of plasmid pZIP-IN (Figure 3). This plasmid contains a chimeric
30 gene including a DNA segment from a strain of *Salmonella* fused with a segment of the alkaline phosphatase gene lacking signal and expression sequences. When expressed in a suitable host cell, the fusion product is localized to the inner membrane/periplasmic space of the host cell.

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Preparation of p-ZIP-IN

pZIP-IN, Figure 3, is a derivative of pBR322 containing a *Bam*HI fragment encoding alkaline phosphatase activity and kanamycin resistance inserted at the *Bam*HI site. The *Bam*HI fragment was cloned from a chromosomal DNA preparation of the *TnphoA* insertion mutant TAG28, which was constructed by *TnphoA* mutagenesis (see above) of *S. typhimurium* TA2361 (*phoN* mutant derived from LT2).

10

Chromosomal DNA was prepared from 50 ml of overnight growth of TAG28 in L-broth with vigorous shaking at 37°C. The bacterial culture was precipitated and washed once in phosphate buffered saline (pH 7.0). The washed bacterial pellet was resuspended in 10 ml of ice cold ET buffer [10 mM EDTA, 10 mM Tris-HCl (pH 8.0)]. Lysozyme was added to a concentration of 0.1 mg/ml and incubated for 15 minutes at 37°C. 1.2 ml of sarkosyl-pronase solution (10% sarkosyl, 5 mg/ml pronase in ET buffer) was added and the solution was incubated for 1 hr at 37°C. The solution was then extracted 3 times with TE [(10 mM Tris HCl, 1 mM EDTA (pH 8.0)) saturated phenol followed by 3 extractions with chloroform:isoamyl alcohol (24:1). The aqueous phase was transferred to a 50 ml beaker on ice and one-half volume of 7.5 M ammonium acetate was added. Three volumes of ice cold absolute ethanol was gently layered on top of the solution. The chromosomal DNA was precipitated onto a glass rod by gently stirring the solution to mix the interface. The precipitated DNA was rinsed once in 70% ice cold ethanol and dissolved overnight in 2 ml of TE buffer at 4°C. The concentration of DNA was quantitated by measuring the O.D. at 260 nm.

2 µg of TAG 28 chromosomal DNA was digested with *Bam*HI at 37°C for 2 hrs. The solution was extracted once with TE saturated phenol, followed by 2 extractions with chloroform:isoamyl alcohol (24:1). The aqueous phase was

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removed and the DNA precipitated by the addition of 1/10 volume 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol followed by centrifugation in a microcentrifuge. 0.2 μ g of pBR322 DNA was digested with *Bam*HI and prepared for ligation as above. Ligation of the vector DNA (pBR322) and TAG 28 chromosomal DNA was performed by overnight incubation at 4°C in 20 μ l of 1X commercial (Promega) ligase buffer and 2 U of T4 DNA ligase.

pZIP-IN was isolated from the ligation reaction by transformation of subcloning efficiency DH5 α competent cells. 5 μ l of the ligation mixture was added to 50 μ l of DH5 α competent cells and incubated on ice for 30 minutes. Cells were heat shocked for 30 seconds by incubating in a 37°C water bath. Cells were cooled on ice for 2 minutes and 0.950 ml of L-Broth was added to the tube. Cells were incubated for 1 hr at 37°C. Transformants with alkaline phosphatase activity and kanamycin resistance were selected by plating 0.1 ml of the bacterial culture on the L-agar plates containing 50 μ g/ml kanamycin and 40 μ g/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate), followed by overnight incubation at 37°C. The following day, kanamycin resistant colonies were visible and all were blue, indicating the transformants had alkaline phosphatase activity. This was confirmed by alkaline phosphatase assays, Western blotting with monoclonal antibodies to alkaline phosphatase, and DNA sequencing of the fusion joint. Figure 3 shows a partial restriction map of pZIP-IN.

30

EXAMPLE 2

The following example illustrates the construction of pZIP-OUT, Figure 2. The plasmid is constructed from a DNA segment of *Salmonella* and a *PhoA* DNA segment lacking signal and expression sequences. When expressed from a

35

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suitable host cell, the fusion protein is localized to the outer membrane of the host cell.

Construction of pZIP-OUT

5

Genomic DNA was isolated from strain TAP43. A 25 ml culture in LB broth was grown overnight at 37°C with shaking. The cells were harvested by centrifugation, and the pellet washed once in PBS. The washed pellet was resuspended in 10 mls of cold TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). One ml of a 1 mg/ml lysozyme solution was added, and the mixture was incubated in a 37°C water bath for fifteen minutes. After this incubation, 1.2 ml of 10% sarkosyl, 5 mg/ml pronase in TE buffer was added, and incubation continued at 37°C for 1-2 hours, until cell lysis occurred. The lysate was then extracted twice with an equal volume of phenol, once with phenol/chloroform, and once with chloroform. To the final extraction, a half-volume of 7.5 M ammonium acetate was added. The solution was mixed gently and placed on ice. Two volumes of ice-cold absolute ethanol were layered on top of the lysate, and the chromosomal DNA was collected at the interface by spooling on a glass rod. The spooled DNA was rinsed once in 70% ethanol, and then allowed to dissolve off of the glass rod into TE buffer overnight at 4°. The buffer, containing the dissolved DNA, was then ethanol-precipitated. The purified chromosome was collected by centrifugation and resuspended in a small volume of TE buffer. 1-5 µg of the purified DNA was restricted with *Hind*III, and then phenol/chloroform extracted and ethanol precipitated. The sample was collected by centrifugation, the pellet washed once with 70% ethanol, and dried under vacuum.

35

Vector pUC18 was also restricted with *Hind*I, extracted, and precipitated in the same manner. The *Hind*III fragments of the genomic DNA were then ligated

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into the *Hind*III site of pUC18 with T4 DNA ligase. After ligation, the DNA was transformed into competent DH5 α cells and plated on L-agar supplemented with ampicillin and BCIP (5-bromo-4-chloro-3-indolyl phosphate), both at 40 μ g/ml. Blue colonies, indicating the presence of an active alkaline phosphatase fusion in the transformant, were selected and analyzed by restriction mapping. Transformant 43-17 contained a 4.5 kp *Hind*III insert in the pUC18 vector. 3.1 kp of this insert consisted of *phoA* sequences, with the remaining 1.4 kp being derived from *Salmonella* chromosomal sequences.

The identity of this clone as a *phoA* fusion was confirmed not only by restriction analysis, but also by Southern blotting, Figure 4, and sequencing. The *Salmonella-phoA* fusion contained within this *Hind*III fragment was designated as the pZIP-OUT cassette. This cassette was subsequently cloned into the *Hind*III sites of the vectors pBR322 and pAT153. The general structure of pZIP-OUT is shown in Figure 2.

EXAMPLE 3

The following example illustrates how DNA may be fused to the gene segments of plasmid pZIP-IN, shown in this example with a portion of the cholera subunit B gene.

Construction of pIMB13

pIMB13 is a derivative of pZIP-IN in which the final 294 base pairs of *ctxB* have been inserted in frame with the *phoA* gene sequence at the *Ssp*I site. The inserted fragment containing the *ctxB* gene sequence is from pRIT10810 which encodes the entire *ctxB* gene. First, the *Ssp*I site in the pBR322 portion of pZIP-IN was eliminated as follows. 2 μ g of a plasmid preparation of pZIP-IN was

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digested with *ScaI* and *EcoRV*. Both enzymes cut at a single site within the pBR322 portion of the vector and generate compatible blunt ends. The digested DNA was precipitated and ligation was performed in 20 μ l of 1X
5 ligase buffer containing 1 U of T4 DNA ligase overnight at 4°C. DH5 α frozen competent cells were transformed with 5 μ l of the ligation reaction mixture.

Transformants were selected on L-agar plates
10 containing 50 μ g/ml kanamycin. Colonies were then replicated to L-agar plates containing 40 μ g/ml ampicillin. Loss of ampicillin resistance encoded by pZIP-IN indicated that the segment from *ScaI* (3844) to *EcoRV* (185) which contained the *SspI* site (4168) had been
15 eliminated. The resulting plasmid pAS28-1 contained a single *SspI* site in the *phoA* sequence which generates an in-frame blunt end cut.

pIMB13 was constructed from pAS28-1 as follows. The
20 *ctxB* sequence encoded by pRIT10810 contains an *SspI* site which generates an in-frame blunt end cut near the 5' end of the structural gene. pRIT1080 also contains an *SspI* site in the pBR322 portion of the vector. Digestion of pRIT10810 with *SspI* generates 2 fragments, one of which
25 contains the 3' final 294 base pairs of *ctxB*. 2 μ g of pAS28-1 and 2 μ g of pRIT10810 were digested with *SspI*. Following phenol/chloroform extraction, the samples were combined and precipitated with 2 volumes of ethanol. Ligation of the sample was performed in 20 μ l of 1X
30 ligase buffer containing 1 U T4 DNA ligase. DH5 α frozen competent cells were transformed with 5 μ l of the ligation mixture. Transformants were selected on L-agar plates containing 50 μ g/ml kanamycin and 40 μ g/ml BCIP. Colonies harboring pAS28-1 with inserts at the *phoA SspI*
35 site appeared white since insertion interrupted the active *phoA* gene fusion. White kanamycin resistant colonies were picked for isolation and screened for

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expression of a *ctxB* fusion protein by Western blotting of total envelope fractions with affinity purified anti-*ctxB*. A DH5 α strain harboring a derivative of pZIP-IN encoding a *ctxB* gene fusion was identified and the
5 plasmid was designated pIMB13.

EXAMPLE 4

The following example is an example of a tripartite
10 fusion prepared from plasmid pZIP-OUT. This plasmid may be used to express a fusion polypeptide from suitable host cells. The DNA inserted in this example is a segment from cholera B toxin subunit.

15 Construction of pRSP18

The construction of the trihybrid fusion, pRSP18, was accomplished as follows. Plasmid pRIT10810, containing the cholera toxin B gene, was first restricted
20 with *EcoRI* and *PstI*. The ends generated by these restrictions were repaired with Klenow, and the vector was ligated back together. This created a .8 kp deletion in pRIT10810, eliminating an undesirable *SspI* site in the vector. This deleted pRIT10810 was then restricted with
25 *HindIII* and *SspI*. pZIP-OUT (in vector pUC18) was doubly restricted with *HindIII* and *PvuII*. A 2.0 kp fragment generated from this double restriction, consisting of 1.4 kp of *Salmonella* sequence and .6 kbp of *phoA*, was isolated and purified after agarose gel electrophoresis.
30 This 2.0 kp fragment was then unidirectionally ligated into the *HindIII*/*SspI* digested pRIT10810. This generated an in-frame fusion of the *Salmonella-phoA* sequences to the *ctxB* sequence (pSP-18). This clone was selected for on the basis of weak tetracycline resistance (1 μ g/ml in
35 L-agar). To make further manipulations of the plasmid more efficient, a kanamycin gene block (Pharmacia) was

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cloned into the *Bam*HI site of PSP18, resulting in the plasmid construction pRSP18.

EXAMPLE 5

5

This example illustrates the procedure for extracting and separating bacterial membranes. After isolation of the membrane fragments, they were analyzed for localization of fusion peptides.

10

Preparation of Bacterial Membranes (Total Envelope) and Separation into Inner and Outer Membrane Fractions

100 ml of overnight bacterial cultures grown in L-
15 Broth with vigorous shaking were pelleted and washed 1X in phosphate buffered saline (pH 7.0). Washed pellets were resuspended in 3 ml of membrane isolation buffer [10 mM NaPO₄, 0.5 mM MgSO₄ (pH 7.0)]. Samples were sonicated for 20 seconds 3 times with cooling on ice in between.
20 Unbroken cells were removed by centrifugation at 7,000 rpm in a Beckman ultracentrifuge SW55 rotor for 1 hr. The supernatants were removed and total envelope pellets were rinsed 1X in sterile deionized water. Pellets were resuspended in 40 μ l of sterile deionized water. One-
25 half (20 μ l) was saved for Western analysis of the total envelope. A 5% solution of sarkosyl in sterile deionized water was added to the remaining 20 μ l to a final concentration of 0.5%. The samples were incubated for 30 minutes at room temperature and centrifuged in a
30 microcentrifuge to pellet the non-soluble fraction representing the outer membrane. The supernatant was removed for Western analysis of the inner membrane fraction. The outer membrane pellet was rinsed once in sterile deionized water and saved for Western analysis.
35 Figure 4 shows immunoblot analysis of membrane preparations using mouse anti-alkaline phosphatase.

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EXAMPLE 6

The following example describes the analysis of alkaline phosphatase activity. For the purposes of the present invention, alkaline phosphatase assays were performed to test for enzyme activity in membrane fractions of host cells in which alkaline fusion proteins were expressed.

10 Alkaline Phosphatase Assays

Alkaline phosphatase activity encoded by pZIP-IN and pZIP-OUT was confirmed by spectrophotometric assay using the chromogenic alkaline phosphatase substrate para-nitrophenol phosphate (PNPP). One ml of overnight bacterial cultures was pelleted for 15 seconds in a microcentrifuge. The pellet was washed once in 1 M Tris-HCl (pH 8.0) and resuspended in 1 ml of 1 M Tris-HCl (pH 8.0). The O.D. 600 of the bacterial suspension was recorded. 50 μ l of chloroform and 50 μ l of 0.1% SDS were added to permeabilize the cells. Samples were vortexed briefly. 0.1 ml of a 0.4% solution of PNPP in 1 M Tris-HCl (pH 8.0) was added and samples were incubated at 37°C. After significant yellow color was observed, 10 μ l of 2.5 M KPO_4 , 0.5 M EDTA was added and samples were placed on ice to stop the reaction. Cellular debris was removed by centrifugation in a microcentrifuge. O.D. 420 of the samples were recorded. The units of alkaline phosphatase activity were calculated by the following formula:

Units activity =

$$1,000 \times \text{O.D. 420} / \text{time of reaction (minutes)} \times \text{O.D. 600}$$

35 Figure 5 shows an immunoblot analysis of urea extracts using anti-alkaline phosphatase as the primary antibody. No reaction is shown with plasmid pBR322 or with plasmid

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pZIP-IN. A reaction is shown with plasmid pZIP-OUT, indicating extraction of the alkaline phosphatase fusion protein.

5 **EXAMPLE 7**

The following outlines the general procedure for extracting proteins from bacterial cells.

10 Urea Extraction of Bacterial Cells

Ten ml of overnight stationary phase bacterial cultures grown in L-Broth with vigorous shaking were cooled on ice for 10 minutes and pelleted at 7,000 rpm in a Beckman J2-21 (JA-17 rotor). The bacterial pellet was washed 3 times in phosphate buffered saline (pH 7.0). The washed pellet was resuspended in 0.1 ml of 6 M urea containing 10 mM Tris-HCl (pH 7.5) and 5 mM EDTA. The suspension was incubated for 20 minutes on ice. Bacteria were pelleted in a microcentrifuge for 1 minute. Centrifugation of the supernatants was repeated to remove any traces of debris. Supernatants were frozen and 20 μ l aliquots were used for SDS-PAGE and Western analysis.

25 EXAMPLE 8

The following example illustrates the expression of a *ctxB* polypeptide from an attenuated *Salmonella* strain with localization of the *ctxB* to the surface of the outer cell membrane.

Preparation of Surface Expressed Cholera Toxin Subunit B

The tribrid fusion in pRSP18 contains a 1.4 kb *Salmonella* DNA sequence which includes expression export signals, Figure 6. The *phoA* sequence of the fusion includes approximately 0.6 kb from the *phoA* fusion joint

-40-

(FJ) to the inframe insertion (IF) of *ctxB*. The *ctxB* sequence includes the final 294 base pairs of *ctxB* beginning at the inframe insertion site IF. Expression and export result in a 32 kDa tribrid fusion protein including the final 98 amino acids of *ctxB* at the C terminus which localizes to the outer membrane. The tribrid fusion in a pIMB13, Figure 7, contains a 1.3 kb *Salmonella* DNA sequence which includes the expression and export signals of the expressed gene. The *phoA* sequence of the fusion includes approximately 0.2 kb from the *phoA* fusion joint FJ to the inframe insertion IF of *ctxB*. The *ctxB* sequence includes the final 294 base pairs of *ctxB* beginning at the inframe insertion site IF. Expression and export result in a 32 kDa tribrid fusion protein including the final 98 amino acids of *ctxB* at the C terminus which localizes to the inner membrane. Figure 8 is a schematic representation of the fusion products.

Whole *Salmonella* TA2362 cells harboring pRSP18 were shown to express cholera B subunit on the outer surface membrane. Antisera to cholera toxin B subunit were prepared. Agglutination of TA2632 harboring pRSP18 was obtained. No agglutination was observed with strain TA2362 alone.

25

An immunoblot analysis of the membrane preparations was run using affinity purified rabbit anti-CTB. *S. typhimurium* TA 2362 harboring pRSP18 showed a 32 kDa CTB tribrid fusion protein in the total envelope (TE). Upon separation of the inner and outer membrane by treatment with 0.5% sarkosyl, the majority of the fusion protein was observed associated with the outer membrane (OM). TA 2362 harboring pIMB13 showed a 32 kDa CTB fusion protein in the total envelope (TE). Upon separation of the inner and outer membrane by treatment with 0.5% sarkosyl, the majority of the fusion protein was found associated with the inner membrane (IM). All lanes were loaded with

-41-

membrane preparations prepared from an equivalent number of cells.

EXAMPLE 9

5

The following example illustrates the procedures contemplated as useful for creating an immune response in a mammal elicited with virulence attenuated *Salmonella* strains expressing antigens on the surface of the intact
10 cell. In this example, CTB is used as an illustration.

Immunogenic Responses from Surface-Expressed CTB

All immune response experimentation will be
15 conducted using CTB responding C57B/6 mice (15,16). An virulence attenuated *S. typhimurium aroA phoN* strain will be utilized in all experiments. Groups of 10 mice/condition will be challenged with the following:
20 *Salmonella* alone, or *Salmonella* with cytoplasmically-encoded CTB (pRIT108010), or inner (pIMB-13, Fig. 7) or outer (pRSP-18, Fig. 6) membrane-expressed tribrid fusion encoding strains. I.P. challenge (5×10^5 cfu) and oral challenge (5×10^8 cfu) will be evaluated. These challenge
25 doses are expected to give optimal results but may require adjusting as necessary. Boosting will be 10 days post-challenge. Mucosal and serum anti-CTB levels will be determined after 1° and 2° challenge by ELISA (15,16) and by the ability to neutralize cholera toxin activity on adrenal cells (1). It will also be determined if the
30 membrane-expressed CTB tribrid polypeptide retains its potent mucosal adjuvant activity (17) by comparing antibody titers to *Salmonella* and *Salmonella* expressing CTB. Since CTB mediates Ig class switching, we will also determine IgA/IgG ratios between the different challenge
35 protocols by ELISA (17). Alternatively, the adjuvant activity of membrane expressed CTB will be evaluated using a purified antigen (i.e., ovalbumin) (18) for

-42-

concurrent challenge with *Salmonella* or *Salmonella* expressing CTB strains. Additional experiments to further characterize adjuvant activity will be performed as indicated.

5

EXAMPLE 10

This example illustrates a contemplated method of inserting a fragment of HIV gp160 gene into plasmid pZIP-OUT of Example 2.

10

Construction of pZIP-OUT Encoding a 60 kDa Fragment of HIV gp120

A clone containing a 3.1 kb *SalI* - *XhoI* fragment encoding the HIV gp160 gene has been obtained. The coding regions of gp120 and gp41 are indicated by the arrow in Figure 10. *PvuII* digestion of this fragment will yield a 1.8 kb fragment which deletes 0.7 kb of gp120 coding sequence. The 4.5 Kb pZIP-OUT cassette, bounded by *HindIII* sites, has been cloned into the *HindIII* site of vector pAT153 (Δ *PvuII* site). This construction has been designated pZIP-OUT-2. pZIP-OUT-2 will be digested with *PvuII* and *SalI*, and the *PvuII* - *XhoI* HIV fragment ligated into these sites. The tribrid fusion polypeptide predicted from this construction will yield a 82 kd polypeptide (2000-4000 dal, *Salmonella*: 20,000 dal, *phoA*: and 60,000 dal, Δ gp120/gp41).

15

20

25

30

The predicted DNA sequence across the *phoA* fusion junction into gp120 is shown in Figure 10. The *phoA*::gp120/gp41 reading frame is indicated by the brackets. The amino acid sequence across the fusion joint is shown.

35

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EXAMPLE 11

The following outlines general protocols for sequencing.

5

Preparation of Templates

pZIP-IN, pZIP-OUT, and pRSP18 were sequenced by the Sanger dideoxy protocol for double stranded DNA
10 templates.

Purified plasmid preparations for sequencing were prepared as follows:

- 15 1. Each strain was grown overnight in 5 ml of LB broth (containing the appropriate antibiotic) at 37°C with vigorous aeration.
- 20 2. The cultures were harvested by centrifugation. The cell pellets were resuspended in 100 µl of 50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0, and incubated at room temperature for 5 minutes.
- 25 3. 200 µl of freshly prepared 0.2N NaOH, 1% SDS were added to each sample. The samples were mixed by inversion, and then incubated 5 minutes on ice.
- 30 4. 150 µl of 3 M potassium acetate (pH 4.8) were added to each sample. The samples were mixed by inversion and incubated for 5 minutes on ice.
- 35 5. The samples were then centrifuged for 5 minutes, and the supernatants transferred to fresh tubes. The samples were centrifuged a second time for 5 minutes and the supernatants transferred as before.

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6. RNase A was added to a concentration of 20 $\mu\text{g/ml}$, and the samples were incubated at 37°C for 20 minutes.
- 5 7. Each sample was phenol/chloroform extracted, chloroform extracted, and then ethanol-precipitated.
8. The DNA precipitates were collected by centrifugation and each DNA pellet was resuspended
10 in 16 μl deionized water, 4 μl 4 M NaCl, and 20 μl 13% polyethylene glycol 8000. The samples were mixed well and incubated on ice for 20 minutes.
9. The samples were centrifuged 10 minutes and the
15 supernatants discarded. The pellets were washed twice in 70% ethanol, dried, and resuspended in 20 μl of dH_2O .

Denaturation, Annealing, and Sequencing of Templates

20

For each DNA template prepared as above:

1. 2 μl of 2 M NaOH, 2 MM EDTA were added to the entire
20 μl sample and the sample was incubated for 10
25 minutes at room temperature.
2. The reactions were neutralized by the addition of
4.5 μl of 2 M sodium acetate (pH 5.0) and 5.5 μl of
distilled H_2O . The samples were mixed well, and then
30 precipitated with 100% ethanol.
3. The DNA pellets were collected by centrifuging for
15 minutes. The pellets were then washed once with
70% ethanol and dried.
- 35 4. All of the following reagents, except primers and
radioactive label, were supplied in the Sequenase

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- sequencing kit, United States Biochemical Co. The dried pellets were resuspended in 7 μ l dH₂O, 2 μ l of 5X Sequenase reaction buffer and 1 μ l (~20 ng) of the appropriate primer. For sequencing the
- 5 *Salmonella* sequences in pZIP-IN and pZIP-OUT, immediately upstream from the *phoA* junction, primer 1(AGA ATC ACG CAG AGC G) was used. For extended sequencing in the *Salmonella* sequences of pZIP-OUT, primer 2 (TTC AGG AAT GCA TGC) was utilized. To
- 10 sequence across the *phoA:ctxB* junction in pRSP18, primer 3(AGC GCG ACC AGT GAA A) was used. The annealing reactions were incubated for 30 minutes at 37°C.
- 15 5. To each annealing mixture, 1 μ l of .1M dithiothreitol, 2 μ l of diluted labelling mix, 1 μ l of [³⁵S]-dATP, and 2 μ l of diluted Sequenase enzyme were added. The reactions were mixed and incubated at room temperature for 5 minutes.
- 20 6. 3.5 μ l of each labelling reaction were then transferred to each termination mixture tube, containing dideoxy ATP, dideoxy GTP, dideoxy CTP, and dideoxy TTP. The chain termination reactions
- 25 were allowed to proceed for 5 minutes at 37°C.
7. 4 μ l of stop solution were added to each reaction, and the reactions were heated to 75°C for 2-5 minutes.
- 30 8. The reactions were loaded onto a 6% acrylamide-urea sequencing gel and electrophoresed at 15 mA for 2-6 hours.
- 35 9. After electrophoresis, the sequencing gel was fixed in 10% methanol, 10% acetic acid, for 1 hour and then dried under vacuum for 1 1/2 hours.

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10. The dried gel was then exposed to autoradiograph film at room temperature for ~16 hours.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: BOARD OF REGENTS, THE UNIVERSITY OF
TEXAS SYSTEM
- (ii) INVENTORS: NIESEL, David W.
MONCRIEF, J. Scott
10 PHILLIPS, Linda H.
- (iii) TITLE OF INVENTION: MEMBRANE EXPRESSION OF
HETEROLOGOUS GENES
- 15 (iv) NUMBER OF SEQUENCES: 2
- (v) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: ARNOLD, WHITE & DURKEE
20 (B) STREET: P.O. Box 4433
 (C) CITY: Houston
 (D) STATE: Texas 77210
 (E) COUNTRY: US
 (F) ZIP: 77210
- 25 (vi) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
30 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: WordPerfect 5.1
- (vii) CURRENT APPLICATION DATA:
- 35 (A) APPLICATION NUMBER: Unknown
 (B) FILING DATE: Unknown
 (C) CLASSIFICATION: Unknown

-48-

(viii) PRIOR APPLICATION DATA:

- 5 (A) APPLICATION NUMBER: 07/792,525 US
(B) FILING DATE: 15 November 1991
(C) CLASSIFICATION: 424

(ix) ATTORNEY/AGENT INFORMATION:

- 10 (A) NAME: KITCHELL, Barbara S.
(B) REGISTRATION NUMBER: 33,928
(C) REFERENCE/DOCKET NUMBER: UTFG111PCT

(x) TELECOMMUNICATION INFORMATION:

- 15 (A) TELEPHONE: 512-320-7200
(B) TELEFAX: 713-789-2679

(2) INFORMATION FOR SEQ ID NO:1:

20 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 213 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
25 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAACCGATT CGCCCCCTTA TAACTATTGT CAGATAACGT TCTGACGGTT 50
30 GTGTAAAAAC ATG GCG CCT CAT TCT TCT GTA GTT GGA GTT AAT 93
Met Ala Pro His Ser Ser Val Val Gly Val Asn
1 5 10

ATG AAA AAA TTT TAT AGC TGT CTT CCT GTC TTT TTA CTG ATC 135
35 Met Lys Lys Phe Tyr Ser Cys Leu Pro Val Phe Leu Leu Ile
15 20 25

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GGC TGT GCT CCT GAC TCT TAT ACA CAA GTA GCG TCC TGG ACG 177
Gly Cys Ala Pro Asp Ser Tyr Thr Gln Val Ala Ser Trp Thr
30 35

5 GAA CCT TTC CCG TTT TGC CCT GTT CTG GAA AAC CGG 213
Glu Pro Phe Pro Phe Cys Pro Val Leu Glu Asn Arg
40 45 50

(2) INFORMATION FOR SEQ ID NO:2:

10

(i) SEQUENCE CHARACTERISTICS:

15

- (A) LENGTH: 387 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

20 GCGTGCATAA TAAGCCCTAC ACAAATTGGG AGATATATCA TGAAAGGCTG 50
GCTTTTCTT GTTATCGCAA TAGTTGGCGA AGTAATCGCA ACATCCGCAT 100
TAAAATCTAG CGAGGGCTTT ACTAAGCTTG CCCCTCCGC CGTTGTCATA 150
25 ATCGGTTATG GCATCGCATT TTATTTTCTT TCTCTGGTTC TGAAATCCAT 200
CCCTGTCGGT GTTGCTTATG CAGTCTGGTC GGGACTCGGC GTCGTCATAA 250
30 TTACAGCCAT TGCCTGGTTG CTTTCATGGGC AAAAGCTTGA TGCCTGGGGC 300
TTTGTAGGTA TGGGGCTCAT AGCTGACTCT TATACACAAG ATGCGCCTGT 350
GACGGAACCT TTCCCGTTTT GCCCTGTTCT GGAAAAC 387
35

-50-

REFERENCES

5 The references listed below are incorporated herein
by reference to the extent that they supplement, explain,
provide a background for or teach methodology, techniques
and/or compositions employed herein.

- 10 Sanchez, J., Johansson, S., Lowenadler, B., Svennerholm,
A.M. and Holmgren, J., Res. Microbiol. 141, 971-979
(1990).
- Strugnell, R.A., Maskell, D., Fairweather, N., Pickard,
D., Cockayne, A., Penn, C. and dougan, G., Gene 88, 57-63
(1990).
- 15 Dougan, G., Hormaeche, C.E. and Maskell, D.J., Parasite
Immunol. 9, 151-160 (1986).
- Sory, M.-P. and Cornelis, G.R., Res. Microbiol. 141, 921-
20 929 (1990).

-51-

CLAIMS:

1. A DNA segment comprising at least a 10 base pair
segment of the DNA sequence of Figure 2 (SEQ ID NO:1),
5 and which will bind to said sequence under high
stringency conditions.
2. The DNA segment of claim 1 which comprises at least
10 a 20 base pair segment corresponding to the DNA segment
of Figure 2 (SEQ ID NO:1).
3. The DNA segment of claim 2 which comprises at least
15 a 30 base pair segment defined in Figure 2 (SEQ ID NO:1).
4. The DNA segment of claim 3 which comprises at least
a 40 base pair segment defined in Figure 2 (SEQ ID NO:1).
20
5. A DNA segment which comprises at least a 10 base
pair segment of the DNA sequence defined in Figure 11
(SEQ ID NO:2).
25
6. The DNA segment of claim 5 which comprises a 20 base
pair segment of the DNA sequence defined in Figure 11
(SEQ ID NO:2).
30
7. The DNA segment of claim 6 which comprises a 30 base
pair segment of the DNA sequence defined in Figure 11
(SEQ ID NO:2).
35

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8. The DNA segment of claim 7 which comprises a 40 base pair segment of the DNA sequence defined in Figure 11 (SEQ ID NO:2).

5

9. The DNA segment of any of claim 1 further defined as encoding an exportation polypeptide capable of localizing a heterologous protein to a bacterial cell membrane.

10

10. The DNA segment of claim 9 wherein the exportation polypeptide is further defined as capable of localizing a heterologous protein to the bacterial cell membrane outer surface.

15

11. The DNA segment of claim 5 further defined as encoding an exportation polypeptide capable of localizing a heterologous protein to to an inner

20

membrane/periplasmic cell space.

12. A DNA segment defined in Figure 2 (SEQ ID NO:1) encoding a 46 kDa polypeptide capable of localizing a heterologous protein to a bacterial cell outer membrane.

25

13. The DNA segment of claim 12 wherein the 46 kDa polypeptide is capable of localizing a heterologous peptide to the outer membrane external surface of the bacterial cell.

30

14. A DNA segment defined in Figure 11 (SEQ ID NO:2) encoding a 55 kDa exportation polypeptide capable of localizing a heterologous protein to an inner membrane/periplasmic space.

35

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15. A recombinant vector comprising the DNA segment of any one of claims 1-13.

5

16. The recombinant vector of claim 15 wherein the DNA segment includes a region encoding an exportation polypeptide, capable of localizing a heterologous protein to an outer membrane of a cell or to the external surface of the outer membrane.

10

17. The recombinant vector of claim 15 wherein the DNA segment includes a region encoding an exportation polypeptide, capable of localizing a heterologous protein to an inner membrane/cytoplasmic space of a bacterial cell.

15

18. The recombinant vector of claim 15 further comprising a gene encoding a desired polypeptide.

20

19. The recombinant vector of claim 18, wherein the desired polypeptide comprises a detectable polypeptide.

25

20. The recombinant vector of claim 19 wherein the gene sequence encoding a detectable polypeptide has at least one restriction site suitable for insertion of a DNA fragment encoding a desired polypeptide.

30

21. A peptide capable of being positioned adjacent to a heterologous protein or peptide and localizing such a protein or peptide in the outer membrane of a cell, said

35

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localizing peptide comprising an amino acid sequence encoded by the DNA segment defined in SEQ ID NO:1.

5 22. A peptide capable of being positioned adjacent to a heterologous protein or peptide and localizing such a protein or peptide in the inner membrane/periplasmic space of a cell, said localizing peptide comprising an amino acid sequence encoded by the DNA segment defined by
10 Figure 11 SEQ ID NO:2.

23. A method of preparing heterologous polypeptides, comprising the steps:
15 preparing the recombinant vector of claim 18;
transforming a cell with said recombinant vector to provide one or more vector-containing
20 recombinant host cells;
selecting a vector-containing host cell clone; and
isolating from said proliferated clone the
25 heterologous polypeptide.

24. A recombinant heterologous polypeptide prepared by the method of claim 23.
30

25. The method of claim 23 wherein the heterologous polypeptide comprises an antigenic protein or an epitope of said antigenic protein.
35

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26. The method of claim 25 wherein the antigenic protein or epitope of said protein is cholera toxin subunit B.

5 27. A *Salmonella typhimurium* transformant prepared by the method of claim 23.

28. A method for preparing a vaccine, comprising the
10 steps:

selecting an antigen or epitopes of said antigen to which an antibody is desired;

15 inserting a gene encoding the antigen or epitopes of said antigen into the recombinant vector of claim 15;

transforming a host cell with said recombinant
20 vector;

screening for transformants;

25 selecting a transformant which expresses the antigen or epitopes of said antigen; and

preparing a vaccine from the cells expressing the antigen or epitopes of said antigen.

30 29. The method of claim 28 further comprising isolating outer cell membrane fractions from said selected transformant for preparing the vaccine.

35 30. The method of claim 28 wherein the host cell is *Salmonella typhimurium* or *Escherichia coli*.

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31. The method of claim 28 wherein the host cell is a virulence attenuated strain of *Salmonella*.
- 5 32. The method of claim 28 wherein the antigen or epitopes of the antigen are bacterial.
- 10 33. The method of claim 28 wherein the antigen comprises cholera toxin B subunit.
- 15 34. A set of primers capable of priming amplification of selected portions of the DNA of claim 4 or claim 8.
- 20 35. A kit comprising at least one cloning vector in accordance with claim 15, the vector being suitably aliquoted into a container.
- 25 36. The kit of claim 35 comprising a first cloning vector encoding an exportation polypeptide capable of localizing to inner membrane/cytoplasmic space of a host cell and a second cloning vector encoding an exportation polypeptide capable of localizing to outer membrane locations of the host cell.
- 30 37. The kit of claim 35 wherein the first cloning vector comprises pZIP-IN or pZIP-OUT.
- 35 38. The kit of claim 35 wherein the second cloning vector comprises pZIP-IN or pZIP-OUT.

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39. The kit of claim 35 wherein the container is a test tube.

5 40. The kit of claim 35 wherein the vector is aliquoted in an amount suitable for convenient use.

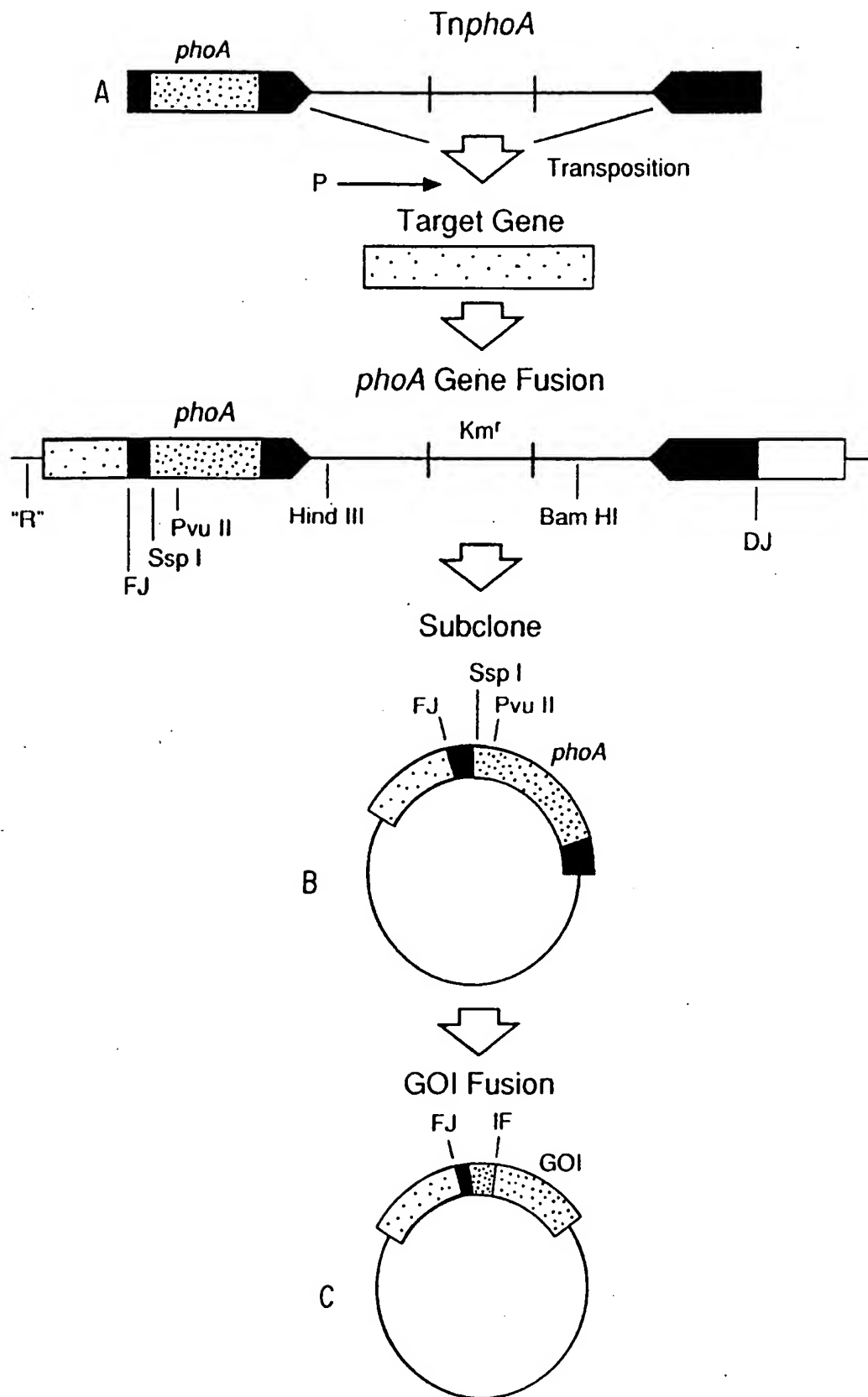


FIG. 1

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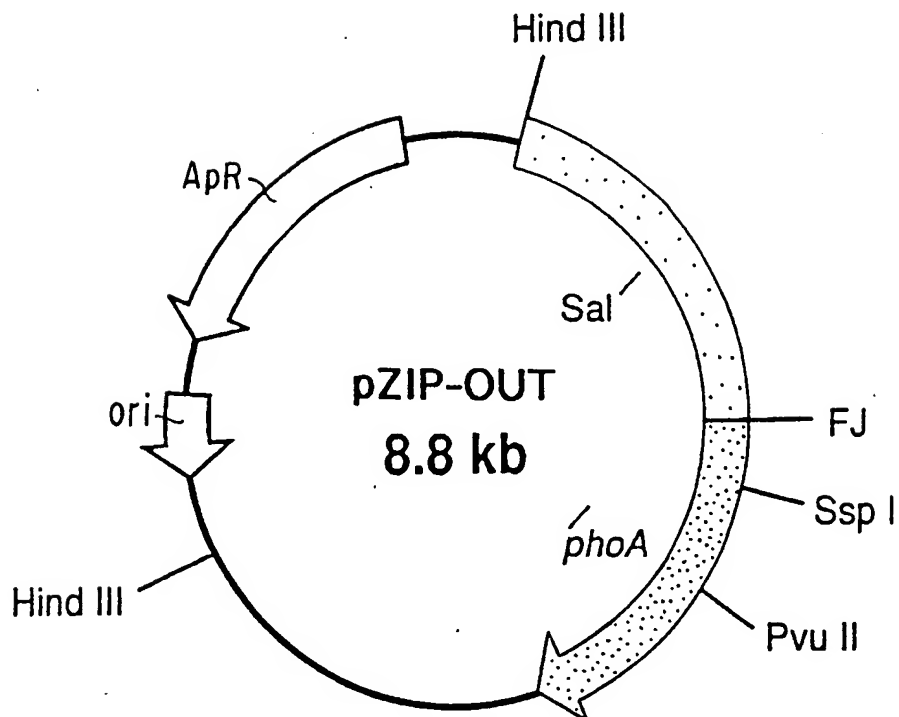


FIG. 2A

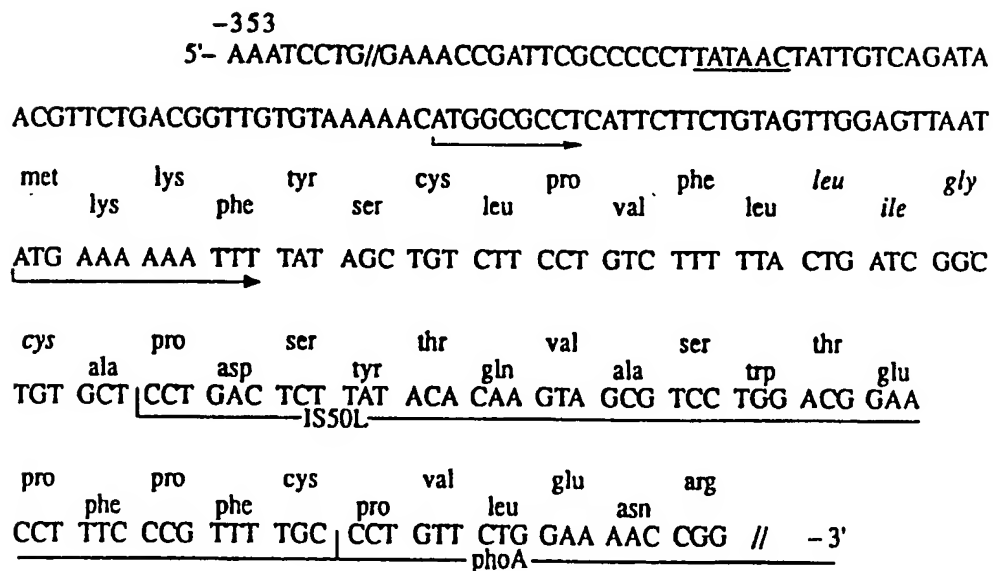


FIG. 2B

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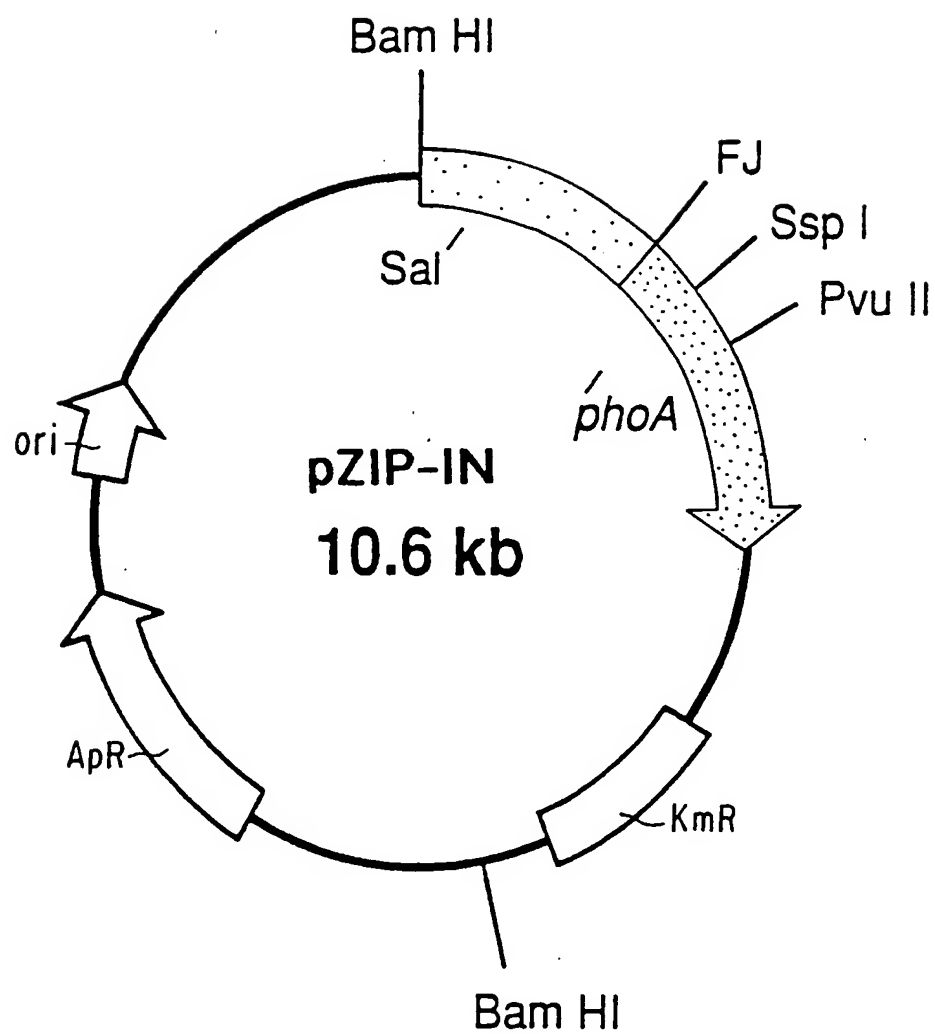


FIG. 3

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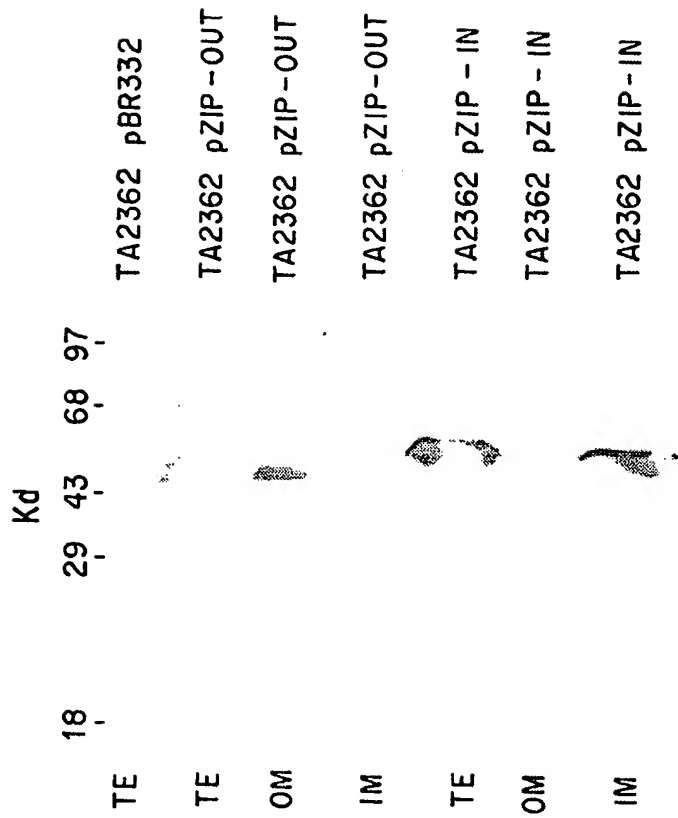


FIG. 4

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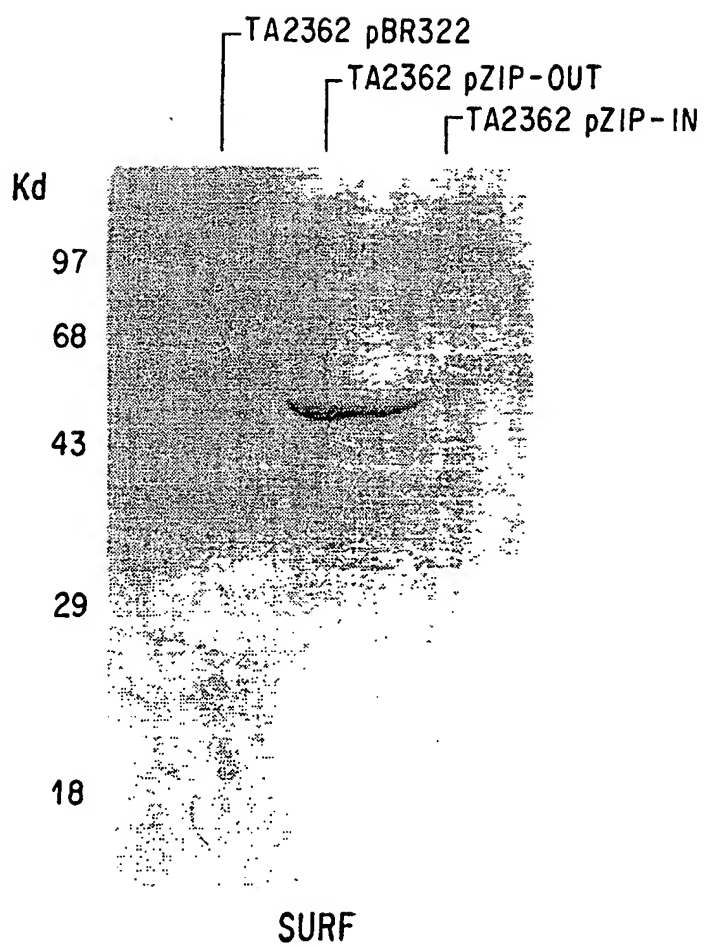


FIG. 5

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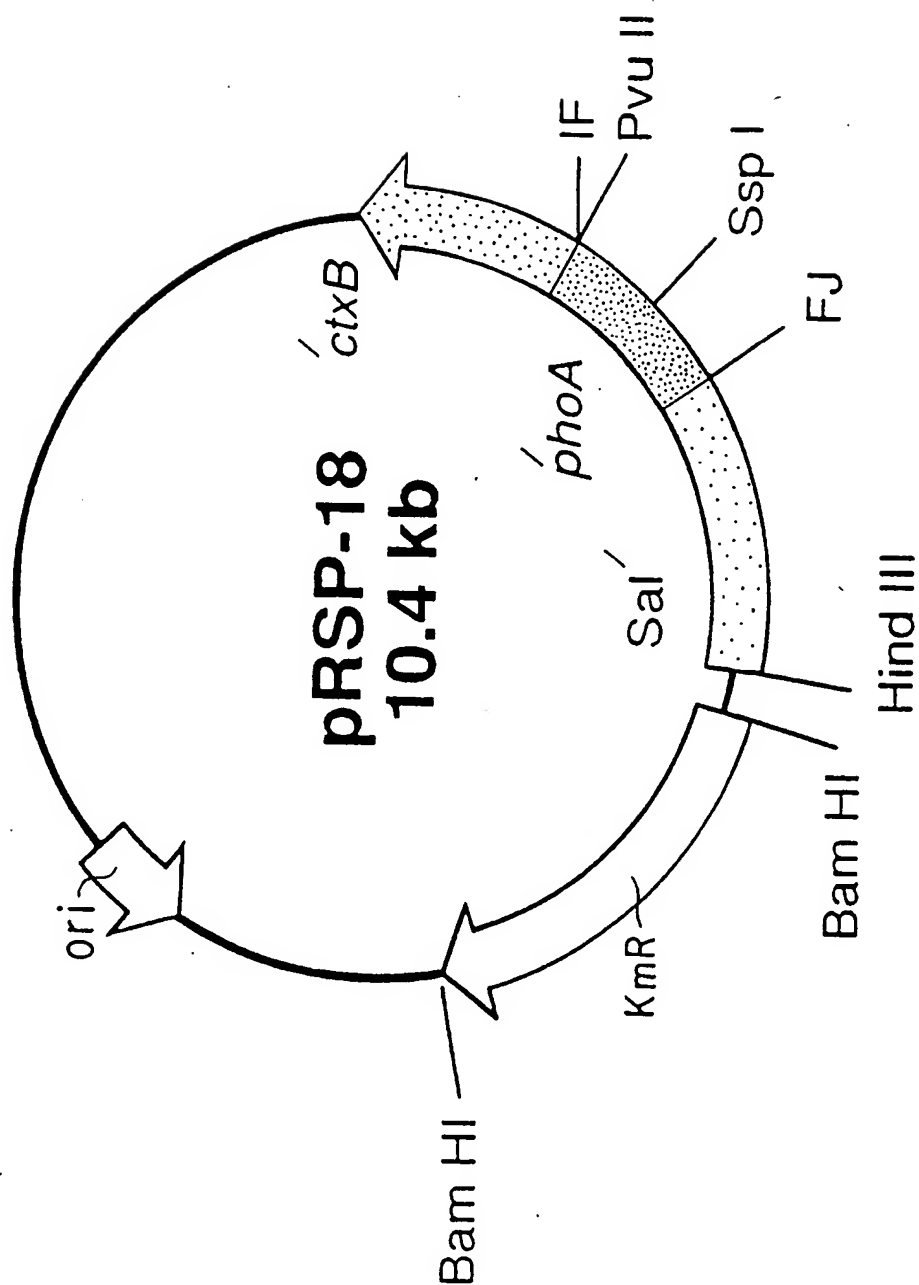


FIG. 6

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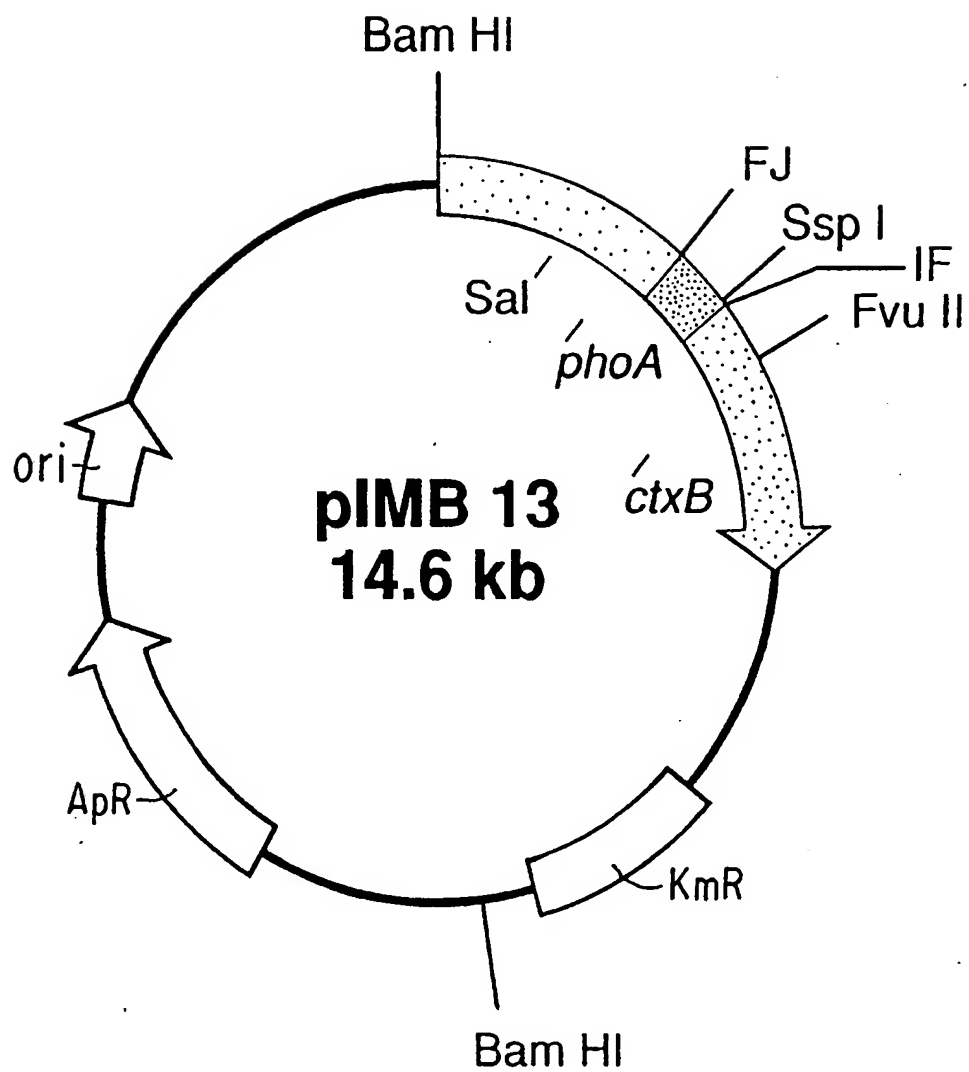
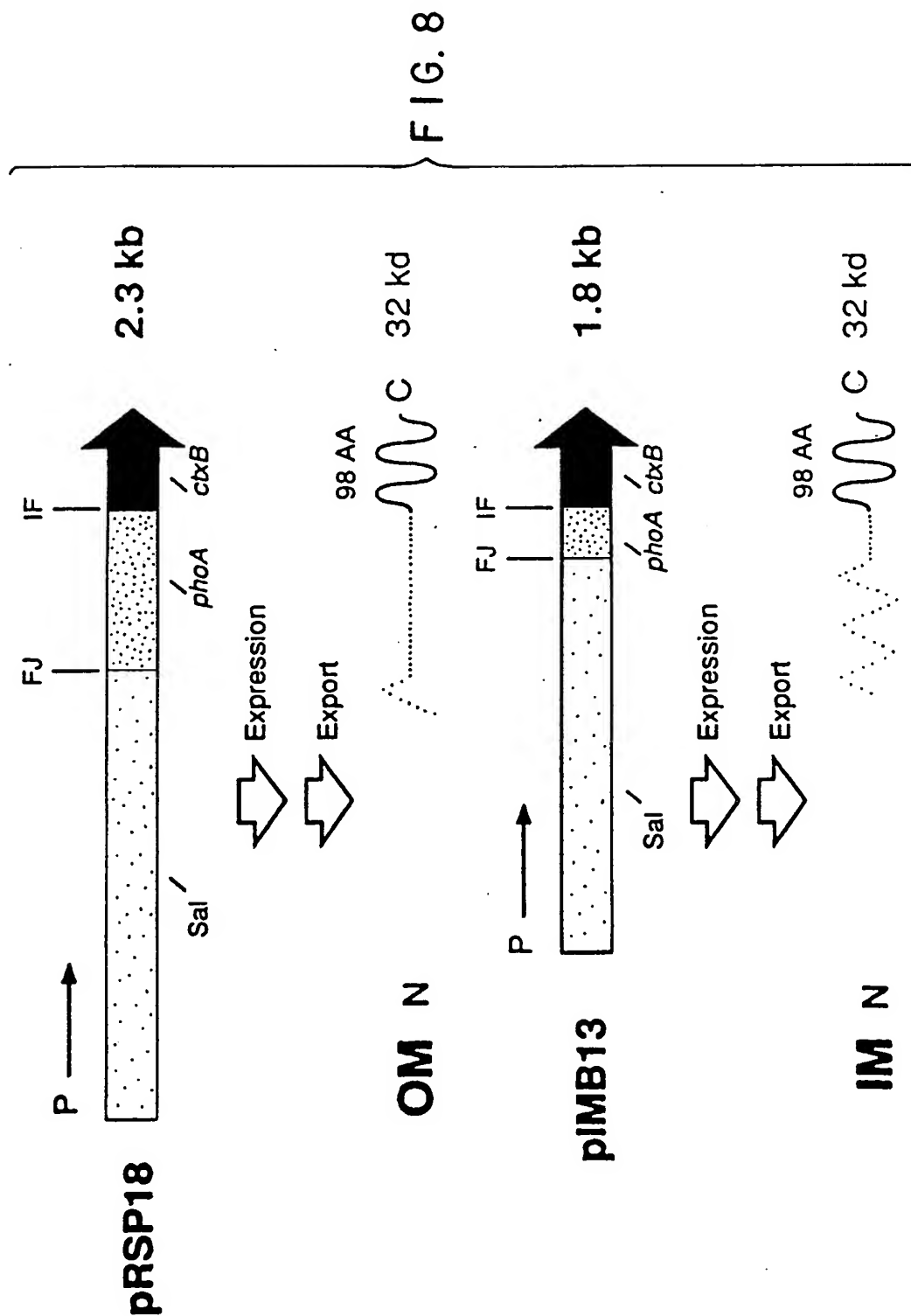


FIG. 7

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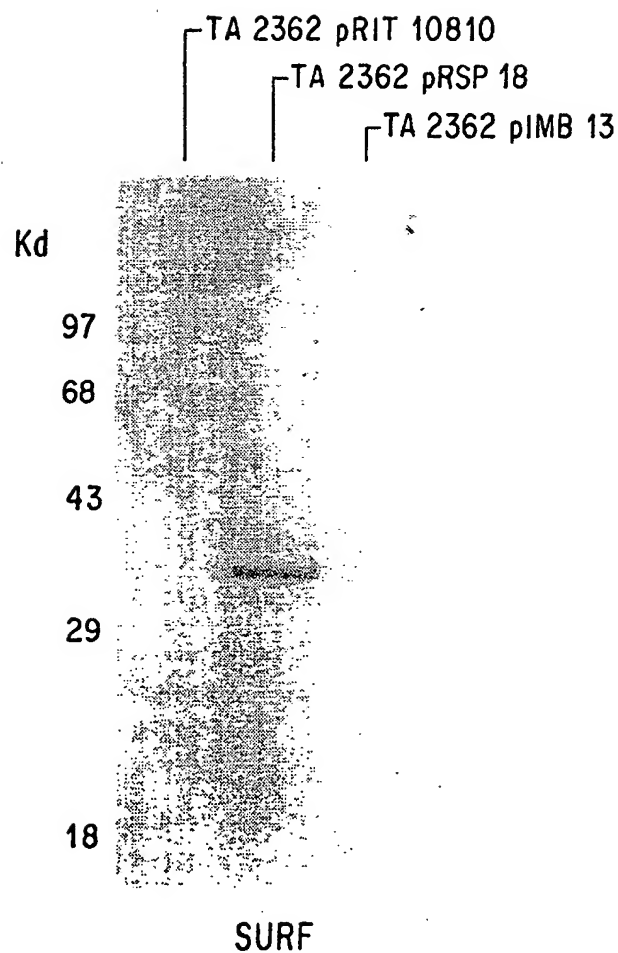


FIG. 9

10/11

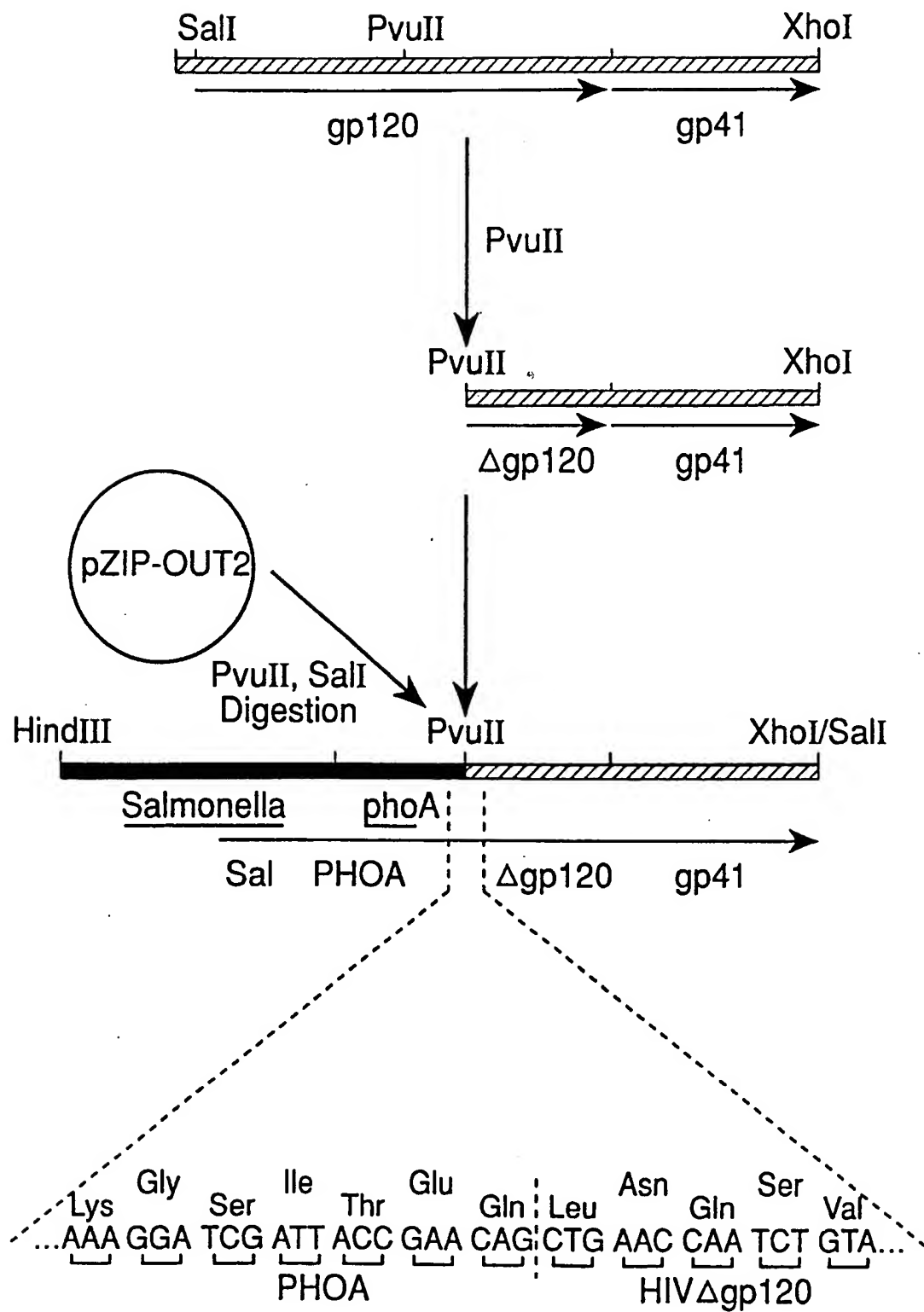


FIG. 10

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5'- GCGTGCATAATAAGCCCTACACAAATTGGGAGATATATC ATG AAA GGC TGG →

CTT TTT CTT GTT ATC GCA ATA GTT GGC GAA GTA ATC GCA ACA TCC

GCA TTA AAA TCT AGC GAG GGC TTT ACT AAG CTT GCC CCT TCC GCC

GTT GTC ATA ATC GGT TAT GGC ATC GCA TTT TAT TTT CTT TCT CTG

GTT CTG AAA TCC ATC CCT GTC GGT GTT GCT TAT GCA GTC TGG TCG

GGA CTC GGC GTC GTC ATA ATT ACA GCC ATT GCC TGG TTG CTT CAT

GGG CAA AAG CTT GAT GCG TGG GGC TTT GTA GGT ATG GGG CTC ATA

GCT GAC TCT TAT ACA CAA GAT GCG CCT GTG ACG GAA CCT TTC CCG
IS50L

TTT GAC CCT GTT CTG GAA ACC -3'
phoA

FIG. 11

SUBSTITUTE SHEET

PCT/US 92/09659

International Application No.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
X	<p>MOLECULAR MICROBIOLOGY vol. 3, no. 12, December 1989, DEVONSHIRE PRESS, TORQUAY, GB; pages 1669 - 1683 H.W. STOKES AND R.M. HALL 'A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons' see page 1675, left column, line 24 - line 28; figure 2</p> <p>---</p>	5-8
X	<p>DE,A,3 901 681 (BEHRINGWERKE MARBURG) 26 July 1990</p>	1-4
Y	<p>see page 2, line 46 - line 55; claims 6-11; table 1</p> <p>---</p>	9,10,12, 13,15, 16, 18-20, 25-33
Y	<p>EP,A,0 368 819 (HOLMGREN,SANCHES CASTILLO) 16 May 1990</p> <p>see page 2, left column, line 1 - right column, line 50; claims 1-15</p> <p>---</p>	9,10,12, 13,15, 16, 18-20, 25-33
X	<p>PROC. NATL. ACAD SCI. vol. 82, no. 23, December 1985, NATL. ACAD SCI., WASHINGTON, DC,US; pages 8129 - 8133 C. MANOIL AND J. BECKWITH 'TnphoA: A transposon probe for protein export signals' see page 8130, left column, line 20 - page 8132, right column, line 43; figures 1,3; table 2</p> <p>---</p>	1-4
Y	<p>see page 8130, left column, line 20 - page 8132, right column, line 43; figures 1,3; table 2</p> <p>---</p>	9,10,12, 13,15, 16, 18-20, 25-33
Y	<p>EP,A,0 407 259 (COMMISSARIAT A L'ENERGIE ATOMIQUE) 9 January 1991</p> <p>see page 1, line 1 - page 4, line 5; claims 1-44</p> <p>---</p>	9,10,12, 13,15, 16, 18-20, 25-33
	---	-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>GENE vol. 104, no. 2, 15 August 1991, ELSEVIER, AMSTERDAM, NL; pages 147 - 153 F. BREITLING ET AL. 'A surface expression vector for antibody screening' see page 147, left column, line 1 - page 149, right column, line 4; figure 1 ---</p>	1-40
A	<p>EP,A,0 355 737 (BEHRINGWERKE MARBURG) 28 February 1990 see page 2, line 1 - page 3, line 8 ---</p>	1-40
P,X	<p>ABSTR. GEN. MEET. AM. SOC. MICROBIOL. vol. 92, 1992, page 225 L. PHILLIPS ET AL. 'Salmonella exposition vectors derived from TnPhoA fusion strains' 92nd general meeting of the american society for microbiology, New Orleans, Louisiana, USA, may 26-30, 1992 abstract H-256 -----</p>	1-27

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9209659
SA 67409

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 16/03/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-3901681	26-07-90	AU-B- 619378	23-01-92
		AU-A- 4862290	16-08-90
		CA-A- 2008244	21-07-90
		EP-A- 0391022	10-10-90
		JP-A- 2247198	02-10-90
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